DNA Aptamers That Bind to PrP^C and Not PrP^{Sc} Show Sequence and Structure Specificity

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DNA aptamers were selected against recombinant human (rhu) cellular prion protein (PrP^C) 23–231 by systematic evolution of ligands *via* a systematic evolution of ligands by exponential (SELEX) enrichment procedure using lateral flow chromatography. The SELEX procedure was performed with an aptamer library consisting of a randomized 40-nucleotide core flanked by 28-mer primer-binding sites that, theoretically, represented approximately 10^{24} distinct nucleic acid species. Sixty nanograms of rhuPrP^C23–231 immobilized in the center of a lateral flow device was used as the target molecule for SELEX. At the end of 6 iterations of SELEX, 13 distinct candidate aptamers were identified, of which, 3 aptamers represented 32%, 8%, and 5% of the sequences respectively. Eight aptamers, including the three most frequently occurring candidates, were selected for further evaluation. Selected aptamers bound to rhuPrP^C23–231 at 10^{-6} *M* to 10^{-8} *M* concentrations. Two of the eight aptamers bound at higher concentrations to rhuPrP^C90–231. Theoretical thermodynamic modeling of selected aptamer sequences identified several common motifs among the selected aptamers that could play a role in PrP binding. Binding affinity to rhuPrP^C23–231 was both aptamer sequence and structure dependent. Further, selected aptamers bound to mammalian PrPs derived from brain of healthy sheep, calf, piglet, and deer, and to PrP^C expressed in mouse neuroblastoma cells. None of the aptamers bound to proteinase K–digested scrapie-infected mouse neuroblastoma cells or untreated PrP-null cells, which further confirmed the PrP^C specificity of the aptamers. In summary, we enriched and selected DNA aptamers that bind specifically to rhuPrP^C and mammalian PrP^C with varying affinities and can be applied to biological samples for PrP^C enrichment and as diagnostic tools in double ligand assay systems. Exp Biol Med 231:204–214, 2006

Key words: DNA aptamers; prion; scrapie; TSE

Introduction

Prion diseases are a group of fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy (BSE) in cows. Pathogenesis of prion diseases involves structural conversion of normal cellular (^{C}) prion protein (PrP) to its pathogenic isoform, PrP^{Sc} (1). PrP^{Sc} has an identical amino acid sequence to PrP^C, however, the biophysical features, such as detergent solubility, secondary and tertiary structures, and stability against enzymatic digestion are dramatically different from PrP^C (1). Proteinase K (PK) digestion of PrP^{Sc} leads to the generation of an infective amino terminus truncated protease-resistant PrP fragment (1).

Safety concerns regarding foods and pharmaceuticals of animal origin have increased since the demonstration that the same agent as BSE is responsible for the devastating outbreak of variant Creutzfeldt-Jacob Disease in humans (2). Monitoring infectious agents in the food chain and pharmaceuticals requires sensitive and reliable analytical procedures capable of quantifying such infectious agents. Definitive diagnosis of prion diseases largely depends on postmortem evaluation of pathognomonic features by anatomic pathology or immunohistochemical staining of the brain sections from affected patients or animals, and

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these procedures may take several days to obtain results. Commercially available diagnostic tests for prion diseases rely on PK resistance of PrP^{Sc} and immunochemical reagents raised against PrP. Several attempts have been made to generate immunologic reagents that detect conformational epitopes of PrPs and differentiate PrP^{Sc} from PrP^{C} (3–5). Recently, a recombinant antibody fragment directed against an epitope that is exposed only in the PrP^{C} conformation but masked in the PrP^{Sc} isoforms has been developed and commercially used in prion disease diagnosis (3). However, this technique requires a competitive assay format and only indirectly measures binding. Despite this progress, there is a desperate need for a repertoire of conformation-specific ligands that can be applied to detection and differentiation of prion proteins.

Aptamers are single-stranded nucleic acid ligands selected against a specific target molecule for desired functions, such as cell binding, peptide binding, small molecule binding, nucleic acid binding, and catalysis of a variety of chemical reactions (6, 7). Aptamers are selected using an *in vitro* procedure termed systematic evolution of ligands by exponential (SELEX) enrichment (6). The SELEX procedure is initiated with an RNA or DNA library consisting of randomized sequences that provide a vast number of sequence-specific three-dimensional structures (7). Aptamers have been extensively investigated as analytical reagents (8), therapeutic and diagnostic agents (9), and for *in vivo* imaging and drug development (10). An initial aptamer library contains a large diversity of randomized nucleotides. Therefore, the SELEX procedure finds unique sequences that perform a specific task, such as strong binding to a protein (7, 11). Aptamers bind specifically to target molecules with high affinities comparable to those of antigen-antibody complexes (12). In contrast to antibodies, aptamers are convenient to modify chemically and do not require biological systems to produce large quantities. Several specific RNA aptamers selected against recombinant hamster PrP (13) and recombinant human (rhu) PrPs (14, 15) have been described. Recently, Sayer *et al.* (16) investigated functional structures of RNA aptamers specific to recombinant bovine PrP fragments. A 60-bp 3′-truncated RNA aptamer was designed from the 116-bp selected core sequence, and was shown to retain all of the parental helical structures and affinity to PrP, indicating conformational specificity of nucleic acid ligands to their specific target molecules. These studies of prion-specific RNA aptamers laid the foundation for our studies and our approach to select DNA aptamers that were more stable and that could be applied to complex and enzyme-rich matrices, such as foods and body fluids, which are potentially detrimental to RNA molecules. In addition, all RNA aptamers that were selected in that study were derived from *in vitro* converted recombinant PrP^{C}, and no reactivity against mammalian counterparts were shown.

In this study, DNA aptamers were selected against rhuPrP *via* the SELEX procedure, using lateral flow chromatography. We generated a panel of DNA aptamers that bind to recombinant PrP^{C} and immunoprecipitated mammalian PrP^{C} derived from a variety of animal species. Further, these DNA aptamers did not bind to PrP^{Sc} and other neuroproteins.

Materials and Methods

Materials for SELEX. An aptamer library that consisted of a randomized 40-mer DNA sequence flanked by two known 28-mer primer-binding sites: (5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-N_{40}-ATTTCTCCTACTGGGATAGGTGGATTAT-3′: where N_{40} represents 40 random nucleotides with equimolar A, C, G, and T) was synthesized (Integrated DNA technology, Inc., Coralville, IA. The same manufacturer was used to synthesize all primers and aptamers applied in this study). The $rhuPrP^{C}$ fragment consisting of amino acid residues 23–231 ($rhuPrP^{C}$23–231) served as the target protein. A device for lateral flow chromatography (6 mm × 65 mm) consisting of a nitrocellulose (NC) membrane immobilized on a polymer support with an aptamer releasing pad at one end and a wicking pad at the other end, was used as the solid-phase support for the SELEX procedures.

SELEX and Synthesis of Selected Aptamers. The aptamer library was enriched for the selection of specific aptamer candidates against $rhuPrP^{C}$23–231 by SELEX enrichment, using a lateral flow chromatography device. Sixty nanograms of $rhuPrP^{C}$23–231 was deposited as a line at the center of the NC membrane and immobilized by air-drying. The NC membrane was blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). The aptamer library was diluted in PBST containing 1% BSA and applied to the releasing pad. After DNA molecules passed through the NC membrane, the solid phase was washed six times with a high-stringency washing buffer (2.2 g N-cyclohexyl-3-aminopropanesulfonic acid, 11.7 g potassium thiocyanate, 0.2 g NaN_3, 21.3 g Triton X-100, 40 ml of 25× PBS, and 950 ml of dH_2O; pH adjusted to 7.6 with 10 *N* NaOH; dH_2O added to bring volume to 1000 ml). The region of the NC membrane coated with $rhuPrP^{C}$23–231, where the high-affinity aptamers were expected to bind, served as a template for polymerase chain reaction (PCR). Amplification was carried out with a set of primers, of which, one (5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′) was biotinylated at the 5′ end to enable easy removal of the reverse complement orientation of the original library using streptavidin-coated magnetic beads (Promega Co., Madison, WI). Unbiotinylated strands (representing the orientation of the original library) were reused for the subsequent rounds of SELEX. Six iterations of SELEX were performed. Binding specificity and affinity of the sixth aptamer pool were investigated by chemiluminescent dot blot and gel shift analyses. The candidates in the selected aptamer pool after the sixth SELEX were cloned

into TA vectors (TOPO II; Invitrogen Co., Carlsbad, CA), and 50 clones were sequenced. Based on the frequency of common sequences found among 50 clones and the theoretical secondary structures obtained using thermodynamics and mathematical-modeling procedures (17, 18), eight selected sequences were synthesized for specificity and sensitivity evaluation. The synthesized aptamers were 5′ biotinylated to enable detection. The end point concentrations at which aptamers bound to rhuPrP^{C}23–231 were measured using an enzyme-linked immunosorbent assay (ELISA) format; whereby 5′ biotinylated aptamers were incubated in rhuPrP^{C}23–231- or rhu90–231 (rhuPrP^{C} fragment consisting of amino acid residues 90–231)-coated 96-well microtiter plates, followed by detection with neutravidin horseradish peroxidase (HRP) conjugate as described in the section entitled, "End Point Concentration at Which Aptamers Bound to rhuPrP^{C}23–231."

Construction of Truncated Aptamers. Sequences of aptamers derived by SELEX are presented in Table 1. Short aptamers consisting of the randomized region alone in sense and antisense orientations with and without flanking overhangs were constructed and biotinylated at the 5′ end. We chose the most frequently identified aptamer (designated as 3–10), which also showed a greater binding ability to PrP^{C}. This aptamer represented 32% of the sequences identified among 50 clones sequenced after the sixth round of SELEX procedure.

Immunoprecipitation of Mammalian PrPs. PrP^{C} was purified and concentrated from brain tissue from apparently healthy animals (sheep, pigs, white tailed deer, and calves). One part of brain tissue was homogenized in nine parts of lysis buffer (10 m*M* Tris, 150 m*M* NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 5 m*M* EDTA, pH 8.0) containing 1 m*M* phenylmethylsulfonyl fluoride. The brain homogenate was centrifuged at 11,700 *g* for 10 mins, and the supernatant was stored in aliquots at –80°C before use. The monoclonal antibody (mAb), FH11 (TSE Resource Center, Institute for Animal Health, Berkshire, UK), was covalently immobilized onto an agarose gel using the Seize Primary Immunoprecipitation Kit (Pierce, Rockford, IL). The brain supernatant was added to the antibody-coupled gel, and immunoprecipitation was performed as suggested by the manufacturer. The eluted PrP fractions were dialyzed against PBS buffer (pH 7.5) and concentrated using centrifugal filter units (Centricon Centrifugal Filter Units, MWCO 10000; Millipore, Billerica, MS). The concentration of purified PrP was measured by bicinchonic acid protein assay (Pierce). The purified PrP was stored at –80°C before use. A protein profile of purified PrP was generated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by Western blot using a mAb, BG4 (TSE Resource Center, Institute for Animal Health).

Table 1. Sequences of Randomized Regions of Selected Aptamers, Aptamer 3 to 10–Derived Short Aptamers

Selected aptamers	SELEX-enriched randomized sequences[a,b]
SSAP1–2	GGCGATG
SSAP1–4	GCGGATCGTG
SSAP1–6	GTAGCCG
SSAP1–9	GGCTACAGGC
SSAP1–13	TGGCAGCAT
SSAP3–10	GTGGGGCA
SSAP3-10A	GTGAGGCA
SSAP3–24	CGGTGGG
SSAP3–59	TGGCGCGC
SSAP 3 to 10–derived short aptamers	
Sri3–10	GTGGGGCA
Sri3–10 RC	TGCCCCAC
Sri3-10OH	*CTTA*CGGTGGGGCA*ATT*
Sri3-10OHRC	*ATTT*GCCCCACCG*TAAG*

[a] Italicized nucleotides indicate overhangs.
[b] The SELEX-derived aptamers used in this study carried both forward (5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-3′) and reverse primer (5′-ATTTCTCCTACTGGGATAGGTGGATTAT-3′) overhangs.

Dot Blot Analysis. RhuPrP^{C}23–231, rhuPrP^{C}90–231, casein (used as a nonspecific protein), and biotinylated primer alone (as a positive control for the assay) were immobilized as dots on an NC membrane by air-drying for proteins and by UV-linking for nucleotides. The membrane was blocked with 1% BSA in PBST and incubated with heat-denatured biotinylated aptamers from the sixth SELEX enrichment. The membrane was washed three times with PBST and incubated with streptavidin-alkaline phosphate conjugate (Promega). After three washes with PBST, the membrane was equilibrated with a detection buffer (0.1 *M* Tris-HCl and 0.1 *M* NaCl, pH 9.5). A chemiluminescent substrate (CDP-star, ready-to-use; Roche, Basel, Switzerland) was added to the membrane and the signal was detected using a ChemiImager 5500 (Alpha Innotech Corporation, San Leandro, CA), with a chemiluminescent filter, for 5 to 15 mins.

Gel Shift Analysis. Synthesized aptamers (10^{-10} *M* to 10^{-12} *M*) or heat-denatured amplicons of the sixth SELEX aptamer pool were incubated with 1 μg rhuPrP^{C}23–231 for 30 mins at room temperature. The mixture was resolved by 1× 0Tris-borate-EDTA (TBE)–buffered native PAGE. When amplicons of the sixth SELEX aptamer pool were used, the aptamers were directly visualized by ethidium bromide staining. When biotinylated aptamers were used, the aptamers were transferred onto a positively charged nylon membrane (Schleicher & Schuell Inc., Keene, NH) and detected by chemiluminescence techniques, as described above in the Dot Blot Analysis section.

3SDS-PAGE and Detection with Aptamers (South-Western Blot Analysis). Recombinant huPrP^{C}23–231 was separated by SDS-PAGE (19) and transferred to an NC membrane by electroblotting at 60 V

for 2 hrs. The NC membrane was blocked with 0.2% Blocking Reagent (Roche Diagnostics Co., Indianapolis, IN) in PBST, followed by incubation with 10^{-10} *M* selected aptamers for 3 hrs. Binding was detected using chemiluminescence methods as described above in the Dot Blot Analysis section.

End Point Concentrations at Which Aptamers Bound to rhuPrP^{C}23–231. Microtiter plates were coated with 100 ng rhuPrP^{C}23–231 in carbonate buffer (15 m*M* Na_2CO_3 and 35 m*M* $NaHCO_3$, pH 9.6) overnight at 4°C. The plates were washed three times with PBST and blocked with 1% BSA in PBST at 37°C for 2 hrs. Synthesized aptamers were diluted in PBST containing 1% BSA at a final concentration of 1 μ*M*, and serially (10-fold) diluted in the microtiter plates. PBS was used as a control. The plates were incubated at room temperature for 3 hrs, followed by three washes with PBST. The biotin label of the bound aptamers was detected by neutravidin-HRP conjugate (Pierce) diluted 1:1000 in PBST containing 1% BSA. A substrate (3,3′,5,5′-tetramethylbenzidine; Sigma-Aldrich, St. Louis, MO) was added to the plates, and the reaction was stopped by the addition of 5% HCl. The optical density was determined at 450 nm. The end point was defined as the dilution at which the optical density of sample wells exceeded the mean optical density of 12 control wells plus 3 standard deviations. The assay was repeated six times.

Cell Lines. Scrapie-infected mouse neuroblastoma cell line (ScN2a) was purchased from InPro Biotechnology, Inc. (South San Francisco, CA). Mouse PrP-null (PsFF)[1] and PrP^{C}-overexpressing (Mo3F4) lines used in cell blots were constructed in the laboratory of S.A.P. (20).[2]

Cell Blot. Cell blot analyses were performed using standard procedures, as described (21). In brief, cells were grown in Dulbecco's modified Eagle's medium (DMEM; Quality Biological, Inc., Gaithersburg, MD) supplemented with 4 m*M* L-glutamine, 10% fetal calf serum, and 100 U/ml penicillin/streptomycin on plastic cover slips placed in the wells of a 24-well plate in 5% CO_2 at 37°C for 4 days. Cells were blotted onto an NC membrane by applying firm pressure for 30 secs. The NC membrane was air-dried and incubated in a lysis buffer (0.5% deoxycholate, 0.5% Triton X-100, 150 m*M* NaCl, and 10 m*M* Tris-HCl, pH 7.5) with or without 5 μg/ml PK for 1.5 hrs at 37°C. The NC membrane was washed in distilled water and incubated for 20 mins with 5 m*M* phenylmethylsulfonyl fluoride at room temperature. The membrane was immersed in denaturing buffer (3 *M* guanidine isothiocyanate and 10 m*M* Tris-HCl, pH 8.0) for 10 mins, washed three times in water, and blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% nonfat dried milk for 2 hrs. When the assay was performed against native PrP, the NC membrane was blocked in 5% nonfat dried milk without treatment with denaturing buffer. After blocking, the membrane was incubated with the mAbs FH11 (1:5000) or GE8 (1:5000; TSE Resource Center, Institute for Animal Health), or with aptamers (10^{-8} *M*). As a positive control, anti–14–3–3 mAb (1:5000; Upstate Biotechnology, Lake Placid, NY) was used to detect neuroblastoma cells on a NC membrane. mAbs and aptamers were detected with anti-mouse IgG-HRP conjugate (1:10,000) and neutravidin-HRP conjugate (1:1000; Pierce), respectively. A chemiluminiscent substrate (ECL Plus Western Blotting Detection Reagents; Amersham Biosciences Inc., Piscataway, NJ) was added, and signal was captured using the ChemiImager 5500 (Alpha Innotech Corporation), with a chemiluminescent filter, for 5 to 15 mins.

Results

Selection of DNA Aptamers Against rhuPrP. After six rounds of SELEX procedures, the selected aptamer pool demonstrated higher affinity to rhuPrP^{C}23–231 than that of the original aptamer library by gel shift (Fig. 1A) and dot blot analyses (Fig. 1B), indicating that the SELEX procedure selectively enriched for aptamers with higher affinity to rhuPrP^{C}23–231. Therefore, the sixth-round aptamer pool was cloned and 50 clones were nucleotide sequenced. We initiated the SELEX with an aptamer library containing 40 randomized nucleotides, however, as a result of six rounds of the SELEX procedure, the randomized region of selected aptamers became 8–10 bp in length. Three of the selected candidates, designated 3–10, 1–2, and 1–7, represented 32%, 8%, and 5% of the sequences, respectively. Sequences of these aptamers are shown in Table 1. Among all sequences obtained, based on the frequency of occurrence in the 50 sequences and theoretical structures (17), eight sequences were selected for synthesis (aptamer SSAP1–2, SSAP1–4, SSAP1–6, SSAP1–9, SSAP1–13, SSAP3–10, SSAP3–24, and SSAP3–59; Table 1) and PrP-binding studies. Representative structures (17) of the aptamers (Fig. 2) indicated that the selected candidate sequences participated in the formation of stem-loop–like structures.

Aptamer-rhuPrP–Binding Studies Using Denaturing and Nondenaturing Conditions. To investigate the role of secondary structures of the randomized region in PrP binding, short aptamers derived from SSAP3–10 were synthesized and analyzed for their binding to rhuPrP^{C}23–231. A short aptamer designated sri3-10OH that consisted of the aptamer 3–10 randomized region in the correct orientation with trimer and tetramer flanking overhangs bound to rhuPrP^{C}23–231, but other short aptamers (randomized sequence alone, reverse complement of randomized sequence, and the reverse complement of randomized sequence with 3- and 4-bp overhangs) did not demonstrate binding by gel-shift analyses (Fig. 3A and B). A native gel electrophoretic pattern of sri3-10OH showed

[1] Manuscript in preparation by Kanthasamy and Anathram

[2] Manuscript in preparation by Priola and Vorberg

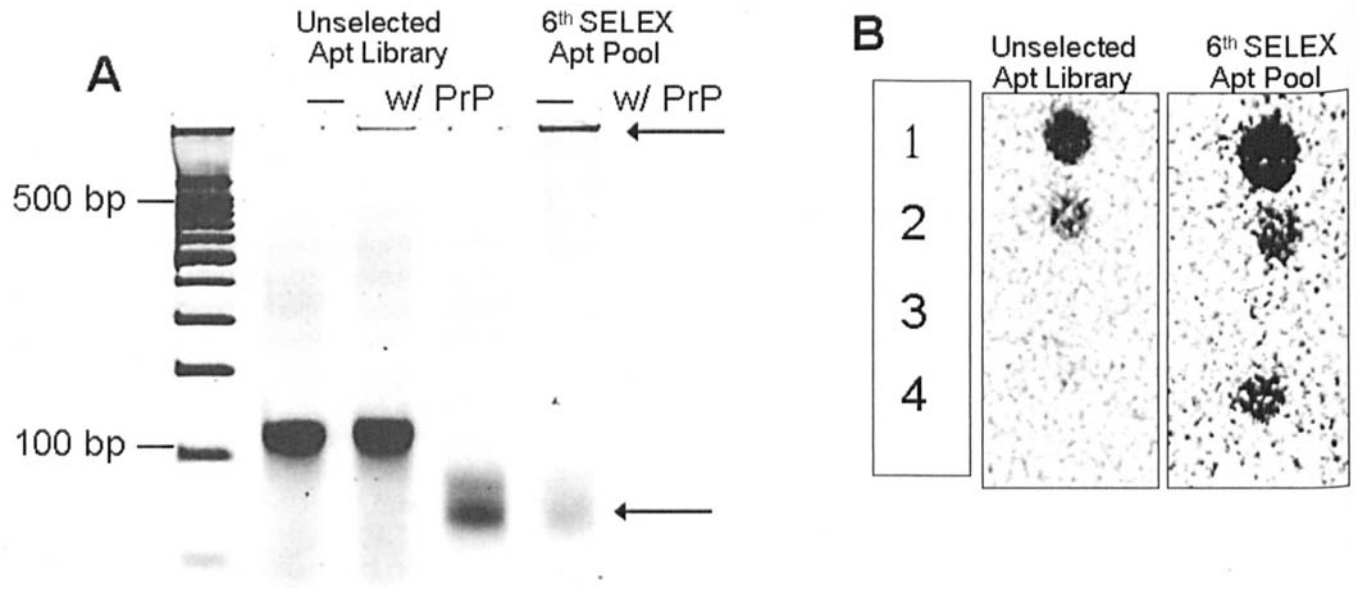

Figure 1. Gel shift (A) and dot blot (B) analyses for unselected aptamer (Apt) library and Apt pool after six rounds of SELEX. (A) Apt alone and Apt-PrP mixture were resolved by nondenaturing PAGE. Apt bands were visualized by ethidium bromide staining. The intensity of the Apt band decreased in the presence of PrP for the selected aptamers (arrow) indicating that the sixth-round SELEX selectively concentrated the Apt species that possessed higher affinities to PrP. (B) Signal from the unselected Apt library was too weak to capture, however, the signal was clear from the selected Apt pool against rhuPrP^{C}23–231, indicating that the selected pool contained Apt with a higher affinity to the target PrP.

multiple bands, suggesting the presence of several secondary structures (Fig. 3A).

All of the selected aptamers showed affinity to rhuPrP^{C}23–231 by gel shift and dot blot analyses (Fig. 4). The dot blot analysis indicated that the aptamers bound to rhuPrP^{C}23–231, but not to rhuPrP^{C}90–231 at 10^{-10} *M*. Selected aptamer candidates also detected denatured rhuPrP^{C}23–231, but not rhuPrP^{C}90–231 in South-Western blots (data not shown).

Binding concentration end points of the selected aptamers to rhuPrP^{C}23–231 measured by a dilution-to-extinction titration method ranged from 10^{-7} to 10^{-8} *M* (Table 2). A single base change of the randomized region of aptamer 3–10 (G to A, designated as 3–10A, Table 1) increased the end point of modified aptamer by 2 logs from

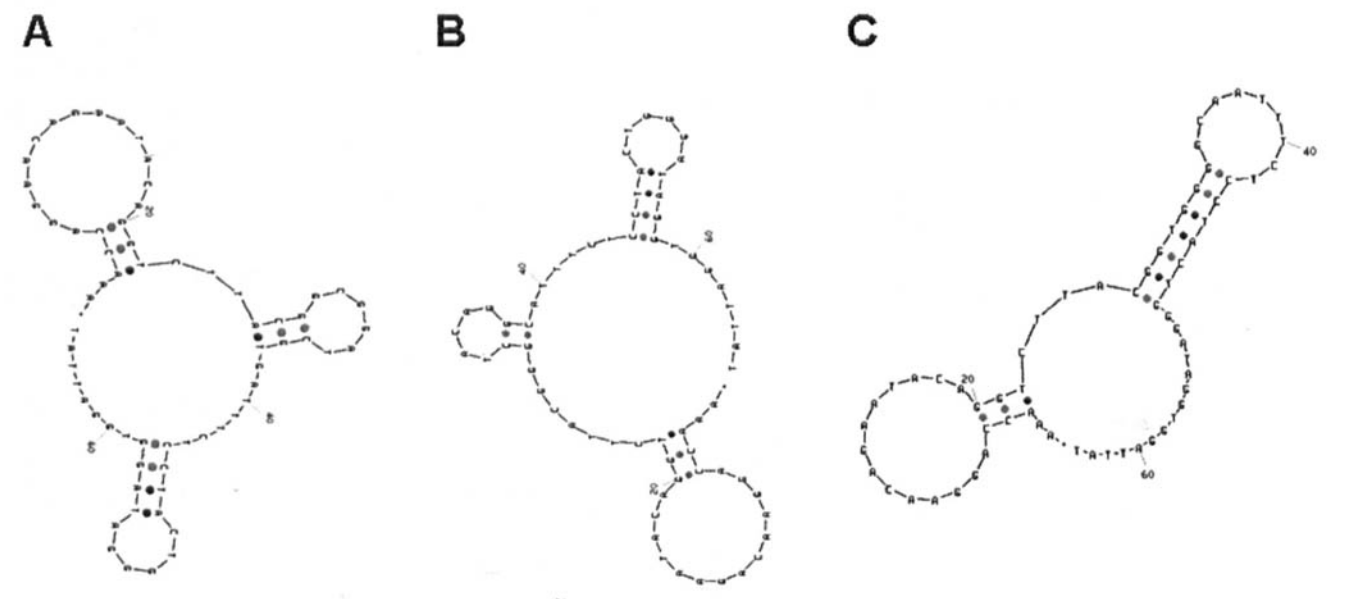

Figure 2. Representative structures of selected aptamers, 1–4 (A), 1–9 (B), and 3–10 (C), derived using the program mfold (17).

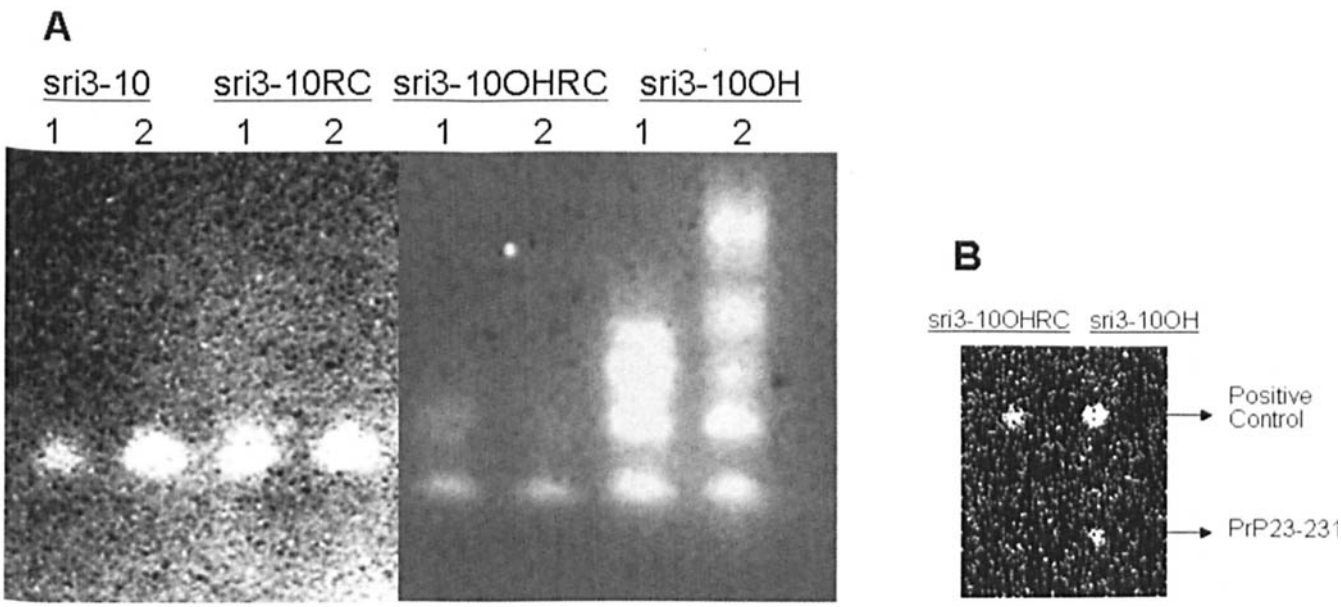

Figure 3. Chemiluminescent gel shift (A) and dot blot (B) analyses for short aptamers. (A) One aptamer alone, two aptamer incubated with rhuPrP^{C}23–231. The short aptamer sri3-10OH demonstrated multiple banding patterns, indicating a presence of secondary structures. In the presence of PrP, the bands shifted to larger molecular sizes for sri3-10OH, indicating that the aptamer bound to PrP, but that other nonspecific short aptamers did not. (B) sri3-10OH also bound to PrP by dot blot analysis, but the reverse complement sequence of sri3-10OH did not detect PrP. The positive control was biotinylated nucleotide.

that of the original aptamer 3–10 (Table 2). Titration to extinction experiments using rhu90–231 indicated that two aptamers bound at 10^{-8} *M* concentrations. The aptamer 3–10 bound to rhu90–231 at concentrations of 10^{-6} *M* and greater.

Selected DNA Aptamers Bind to Mammalian PrPs. Using our panel of DNA aptamers that reproducibly bound to a recombinant PrP at nanomolar concentrations, we investigated their binding abilities to mammalian PrPs enriched by immunoprecipitation of brain extracts and PrP^{C} expressed in cell cultures.

We used immunoprecipitation to purify (SDS-PAGE

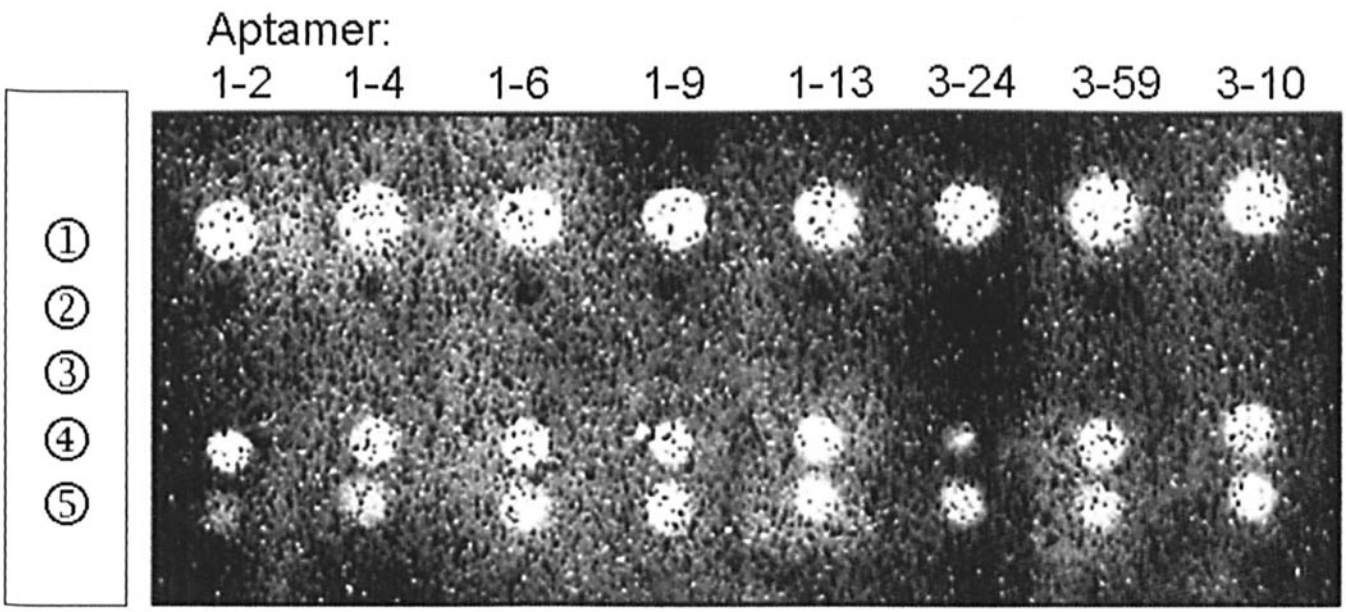

Figure 4. Chemiluminescent dot blot analysis for selected aptamers that bound to PrPs. The left panel indicates positions of immobilized proteins and a control. 1: positive control for the assay (biotinylated nucleotides); 2: nonspecific protein (casein); 3: rhuPrP^{C}90–231; 4: rhuPrP^{C}23–231; and 5: PrP^{C} immunoprecipitated from sheep brain. The selected aptamers bound to rhuPrP^{C}23–231, but not to rhuPrP^{C}90–231, suggesting that the binding sites of the aptamers are located between amino acid residues 23 and 89. The aptamers reacted with recombinant and mammalian PrP^{C}.

Table 2. Binding Concentration End Points of the Selected Aptamers Against rhuPrP^{C}23–231, Measured by Concentration Gradient Titration

Aptamer	End point concentrations against rhu23–231 (*M*)
SSAP1–2	6.3×10^{-8}
SSAP1–4	7.3×10^{-8}
SSAP1–6	6.3×10^{-8}
SSAP1–9	2.5×10^{-7}
SSAP1–13	1.5×10^{-7}
SSAP3–10	1.6×10^{-8}
SSAP3-10A	1.0×10^{-6}
SSAP3–24	3.4×10^{-8}
SSAP3–59	5.2×10^{-8}

and Western blot data not shown) and concentrate PrPs from brain homogenates of healthy sheep, calves, piglets, and deer, with a final concentration of approximately 0.5 mg/ml. All eight selected aptamers bound to immunoprecipitated sheep PrP by dot blot analyses (Fig. 4) and gel shift (representative data for three aptamers are shown in Fig. 5). Although the dot blot analysis was not quantitative, selected aptamers seemed to bind to immunoprecipitated sheep, bovine, porcine, and deer PrPs with varying affinities (Fig. 6).

Selected aptamers bound to mammalian PrP^{C} expressed in Mo3F4 cells as shown (Table 3) when the cells were immobilized on NC membranes. Neither the selected (SELEX derived) nor the simulated aptamers generated any signal against PrP-null cells (Table 3). Anti-PrP mAbs FH11 (Table 3) and GE8 (Table 3) also did not generate a signal against PrP-null cells. We used 14–3–3γ, an intracellular neuroprotein, as a positive control for cell blot analysis, because it is a neuronal protein that is abundant in most areas of central nervous system (22). Anti-14–3–3 mAb gave positive signals in both PrP-null (Table 3) and ScN2a cells (Table 3). The selected aptamers did not bind to 14–3–3 (Table 3) nor to other neuroproteins expressed by PrP-null cells by gel shift, dot blot, and South-Western blot analyses (data not shown). Selected aptamers detected PK-untreated PrP expressed by ScN2a cells (Table 3). The epitope of anti-PrP mAb GE8 is located in the C-terminus of PrP. mAb GE8 detected PrP from PK-treated ScN2a cells (Table 3), indicating that the ScN2a cells expressed PrP^{Sc}. In contrast, mAb FH11 did not generate any signal against PK-treated ScN2a cells in the N-terminus of PrP, because the epitope of FH11 is expected to be degraded (Table 3). Aptamers did not bind to the PK-digested PrP fragments in ScN2a cells (Table 3). PK treatment digested the N-terminus of PrP^{Sc}, therefore, the remaining products were considered to be mostly PrP^{Sc} fragments containing amino acid residues 90–231 (1). This finding concurred with our observation that aptamers bound to recombinant PrP23–231 but not to recombinant PrP90–231.

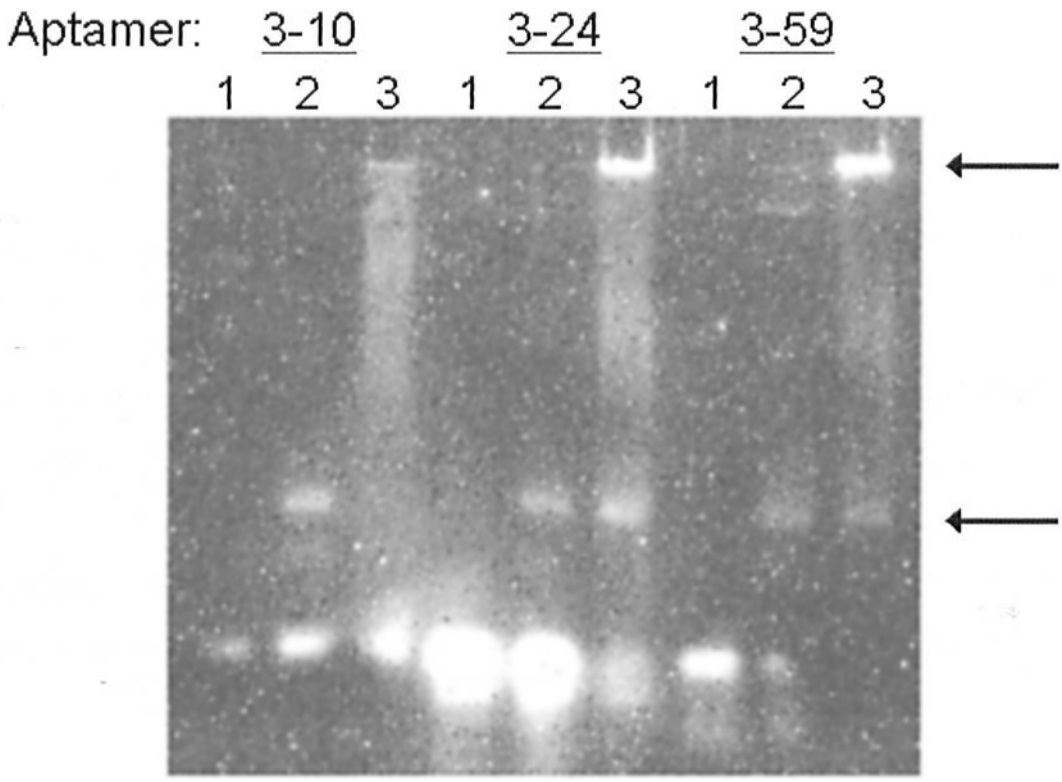

Figure 5. Gel shift analysis showing the affinity of the selected aptamers against recombinant and mammalian PrP^{C}. 1: aptamer alone; 2: aptamer incubated with immunoprecipitated ovine PrP; and 3: aptamer incubated with rhuPrP^{C}23–231. In the presence of ovine PrP and rhuPrP, the aptamer bands shifted to larger molecular sizes (arrow), indicating that the aptamers bound to the PrPs.

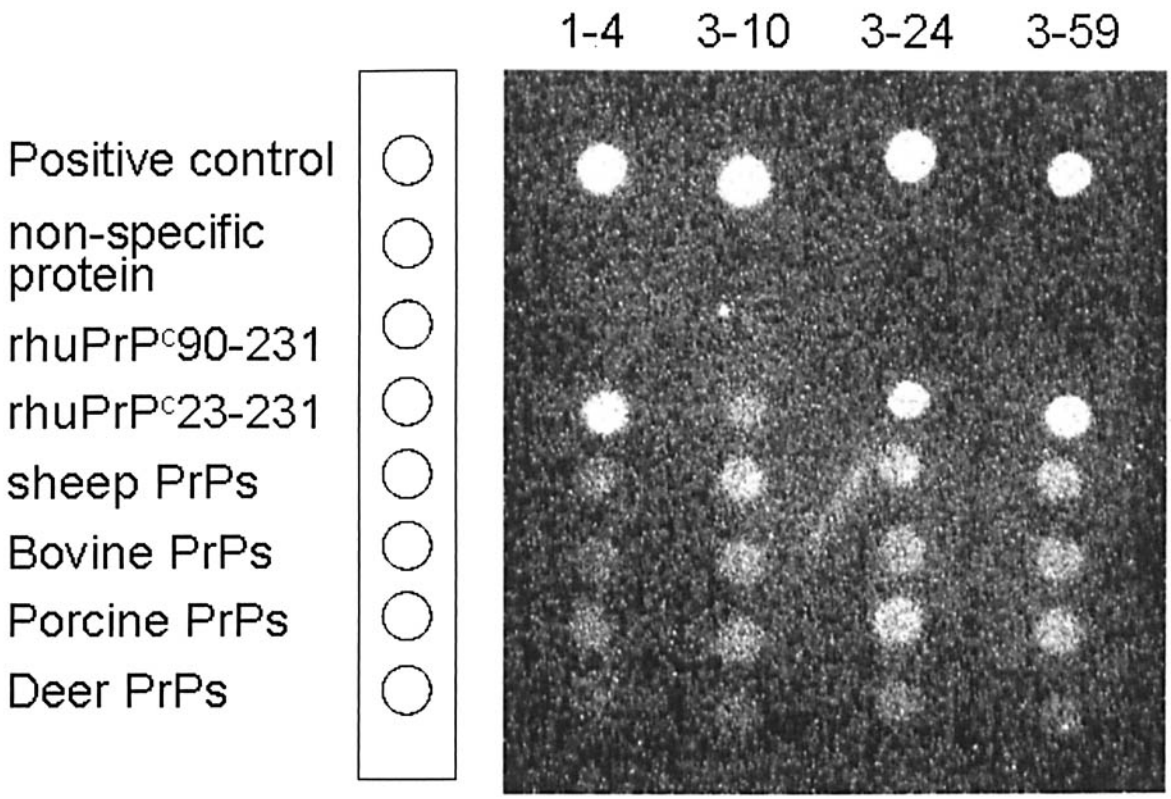

Figure 6. Dot blot analysis with selected aptamers against PrP^C enriched from brain tissues of a variety of animal species. The left panel indicates the positions of the immobilized proteins and a control. The positive control was biotinylated nucleotide. Casein was used as nonspecific protein. A dot of rhuPrP^C90–231 or rhuPrP^C3–231 contains approximately 1 μg of protein. Sheep, cattle, pig, and deer dots contain approximately 2 μg of PrPs derived from brain tissues of apparently healthy animals.

Discussion

This study was undertaken to develop ligands that could potentially differentiate normal and abnormal prion isoforms. Toward this end, we undertook a well-established SELEX protocol to generate a panel of DNA aptamers against PrP^C. We tested their abilities to bind to several segments of PrP as well as normal and abnormal prion isoforms to evaluate their usefulness in diagnostics of prion disease.

Interest in the pathobiology and epidemiology of human and animal prion diseases has recently accelerated

Table 3. Aptamer Binding to PrP^C Expressed on Neuroblastoma Cells, Using Standard Cell Blot Assays[a] Under Varying Conditions

Cell line	Mo3F4 cells[b]		PrP-null cells[c]		ScN2A cells[d]		
						PK untreated	
Conditions/ligand used for blots	Nondenaturing conditions	Denaturing conditions	Nondenaturing conditions	Denaturing conditions	PK treated	Nondenaturing conditions	Denaturing Conditions
SSAP1–6	+	−	−	−	−	+	−
SSAP3–24	+	−	−	−	−	+	−
SSAP3–10	+	−	−	−	−	+	−
SSAP3–59	+	−	−	−	−	+	−
Monoclonal antibody FH-11[e]	+	+	−	−	−	+	+
Monoclonal antibody GE-8[f]	+	+	−	−	+	+	+

[a] All cell blots were performed as described in Ref. 21, with minor modifications.
[b] Mo3F4 cells are PrP^C-overexpressing cells and are described in Ref. 20.
[c] PrP-null cells were a kind gift from Dr. A. Kanthasamy.
[d] ScN2A cells are scrapie-infected neuroblastoma cells that express PrP^{Sc}.
[e] Monoclonal antibody FH-11 was obtained from the TSE Resource Center. This antibody recognizes a motif in the 23–90 region of the PrP molecule and, therefore, does not have any binding to PK-treated PrP^{Sc}.
[f] The monoclonal antibody, GE-8, was obtained from the TSE Resource Center. This antibody binds between amino acid residues 90 and 230 on the PrP molecule and, thus, binds to PK-treated PrP^{Sc}.

for several reasons. First, the mounting experimental evidence has generated great interest in what seems to be a protein-initiated mechanism of disease (23–26). Second, the demonstration that prions are responsible for BSE (27–30), which has infected large numbers of cattle in Great Britain, the recent report of a case of BSE in the United States, and the presence of chronic wasting disease (CWD) in feral and captive deer populations have increased the concern that animal-to-human transmission of prion disease poses a substantial threat to the human race and its food chain; clearly much more effort is needed to prevent this possible epidemic and has lent a new urgency to the quest for accurate diagnostic tools and efficacious therapeutic tools.

PrP^C is a sialoglycoprotein bound to the cell surface through a glycosyl phosphatidyl-inositol anchor. The infectious isoforms or PrP^{Sc} differ from PrP^C in that they are insoluble in nonionic detergents or chaotropic agents and are partially PK resistant (31). Indeed, these characteristics of prions are applied in currently available diagnostics to identify the presence of an infectious form of the prion protein for confirmatory diagnosis in postmortem tissue. Thus, the development of diagnostic tools that are more sensitive in addition to the identification and manufacture of optimal ligands (such as antibodies, receptors, or aptamers) that are able to differentiate prion isoforms will be very useful in generating safe foods and pharmaceuticals. These ligands will also become an integral part of the diagnostic armamentarium of prion disease and prion detection.

Aptamers Enriched by SELEX Bind to Both Recombinant and Mammalian PrP^C and Not to PrP^{Sc}. SELEX-derived aptamers detected rhuPrP^C23–231 when PrP was presented in its native form. Although the aptamers were selected against, and reacted with, a recombinant full-length prion fragment, they also showed affinity to mammalian PrP^C concentrated from brain homogenates and cultured cells. Aptamers, like some currently available antibodies, bind to PrP^C despite the presence of large glycans in mammalian PrP at amino acid residues N181 and N197 (32). Because PrP is a highly conserved protein among animals and humans (1), it may be a challenge to generate antibodies that differentiate PrPs from different species. Our results demonstrate that the selected aptamers detect immunoprecipitated PrP from sheep, calf, piglet, and white-tailed deer, suggesting that species- and isoform-specific DNA aptamers could be selected. These studies are currently underway in our laboratory.

The binding sites of six aptamers identified in this study are located between amino acid residues 23 and 89 of PrP. This finding is congruent with previous studies with RNA aptamers selected against PrPs, which showed that an RNA aptamer selected against recombinant hamster PrP23–231 bound to the PrP fragment containing amino acid residues 23–52 (13). In the presence of a mAb directed against amino acid residues 37–53 of PrP, the RNA aptamer retained its affinity for PrP23–231, indicating that the RNA aptamer interacted with PrP through amino acid residues 23–36 of PrP (13). Another study using rhuPrP to characterize RNA aptamer binding suggested that PrP possessed two RNA binding sites: one was found in the N-terminus between amino acid residue 23 and 90 and the other was in the C-terminal core structure of PrP (15). Although binding of these RNA aptamers to the *in vitro*–derived β-form of the prion was shown, neither its reactivity to mammalian prions nor the specificity to PrP^{Sc} in a background of large amounts of nonspecific host proteins were shown. In contrast, DNA aptamers identified in the current study were able to bind to prions derived from a variety of host species.

Our data indicated that there was good affinity between PrP^C and the selected aptamers, and that the binding was specific to PrP^C in its native form, as demonstrated by the lack of reactivity to other neuroproteins expressed by PrP-null cells. The selected aptamers seem to recognize the N-terminus of PrP, where PrP is rather flexible and lacks defined secondary structures (33). Thus, the data strongly suggest that the selected aptamers were PrP^C conformation specific. These findings parallel those reported by Sayer *et al.* (16) on PrP^{Sc} specific aptamers and indicate that aptamers could be applied to the differentiation of prion conformations. Taken together, the panel of selected aptamers specifically bound to a PrP^C conformation and not to PrP^{Sc} or to other neuroproteins.

Studies on Aptamer-PrP Binding Kinetics Demonstrate Aptamer Sequence and Structure Specificity. Because our analyses of selected aptamers identified similarities in structures of the aptamers and suggested a sequence-structure relationship, we queried the role of their nucleotide sequences and secondary structures in binding to PrP^C.

The role of secondary structures of the randomized region of selected aptamers in PrP-binding was investigated using short aptamers designed from aptamer 3–10. The data suggest that the aptamer secondary structures were necessary for the binding of aptamer to rhuPrP^C23–231. The findings that reverse complement of sri3-OH or other sequences neither showed multiple single-strand conformations nor bound to PrP^C are highly suggestive of sequence and structure specificity in aptamer-PrP^C interactions.

A second set of studies to evaluate binding affinities and the sequence specificity of aptamer-PrP binding showed that swapping one nucleotide (G→A) within the selected region of aptamer 3–10, led to a 2 $\log_{10}$ drop in its binding end point to PrP^C, indicating sequence specificity. In sum, these studies indicate that aptamer-PrP binding was associated with affinities comparable to those of mAbs and that the binding was aptamer-sequence specific.

That the randomized region of our library was 40-bp, but a majority of our selected aptamers was 8–10 bp in length, deserves comment. Taq polymerase, DNA polymerase from *Thermus aquaticus*, has domains responsible for DNA polymerase and 5′ endonuclease activities (34). The

endonuclease activity is structure specific and cleaves single-stranded DNA or RNA at the bifurcated end of a base-paired duplex (34). During PCR, single-stranded DNA generally forms stem-loop–like structures when heated and cooled, conditions that occur between the denaturation and annealing cycles of PCR. These structures are targets of the 5′ nuclease activity of Taq polymerase for cleavage, resulting in reduced lengths of the selected aptamers. Because the DNA polymerase activity is not coupled to nuclease cleavage (34), this issue could be overcome by using the Klenow fragment, a molecule that is an N-terminal deletion mutant of Taq DNA polymerase lacking 5′ nuclease activity (35). Another possible cause for the loss of nucleotides during our SELEX could have been the fact that the initial aptamer library may have contained multiple truncated products, resulting in shorter selected sequences. Nonetheless, the SELEX procedure successfully selected aptamers that specifically recognized recombinant and mammalian PrPs. Smaller randomized regions of the selected aptamers compared with the original library might have reduced its diversity. However, as Sayer *et al.* demonstrated (16), truncated aptamers retained their specific affinity to the recombinant target, indicating that binding ability of aptamers remains as long as its conformational specificity is conserved. This was also consistent in our truncated aptamer studies. Because the selected sequences were parts of stem-and-loop–like structures of the selected aptamers, the sequences might have been conserved during the selection because of their specific conformational binding to PrP^{C}.

PrP^{C} specific aptamers could serve as PrP^{Sc}-enriching reagents or as ligands in competitive transmissible spongiform encephalopathy (TSE) diagnostic assays. Because most antibodies generated to date bind to both PrP^{C} and PrP^{Sc}, a PrP^{C}-specific reagent, such as the aptamers we describe herein, can serve as an adjunct in current diagnostics. For example, a sample could be directly reacted with an antibody without any need for protease treatment if it has already been treated with PrP^{C}-specific aptamers to remove all residual normal prions and, thus, simplifying the diagnostic protocol. Additionally, one could envision the application of these PrP^{C} specific aptamers in the treatment of TSEs. In this case, these reagents could serve to bind PrP^{C} and abrogate PrP^{C}-PrP^{Sc} interactions, inhibiting formation of the β-sheet–rich pathogenic isoforms.

In summary, we generated a panel of aptamers that bind to recombinant and mammalian PrP^{C} and not to PrP^{Sc}. The PrP^{C} specific aptamers seem to recognize a conformation and could be used in competitive or double-ligand assay formats to differentiate prion isoforms, aiding in the diagnostics of TSEs. The PrP^{C}-specific aptamers could also be applied as therapeutic tools to deter the progression of TSEs, and some aptamers developed in these studies may find application in the future to the decontamination of blood, body fluids, foods, pharmaceuticals, and cosmetics in an automated fashion during manufacture. Because selected aptamers seemed to bind to different mammalian PrPs with varying degrees, we anticipate developing an aptamer panel that distinguishes between PrP strains and between isoforms across species. Such ligands are extremely desirable not only to detect and decontaminate pathogenic PrPs but also to accelerate molecular epidemiologic investigations of prion diseases.

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