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Detection of proteins based on amino acid sequences by multiple aptamers against tripeptides

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Abstract

A number of different ligands have been tested in the course of the development of protein array technology. The most extensively studied example of protein ligands has been based on antibody–antigen interaction. Other examples include protein–protein, protein–nucleic acid, and protein–small molecule interactions. All these ligands can recognize and specifically bind to protein epitopes. In this study, we have developed a novel technology using DNA-based aptamers to detect proteins based on their amino acid sequences. Mouse cathepsin D was used for the proof of principle experiment. Four tripeptides, Leu-Ala-Ser, Asp-Gly-Ile, Gly-Glu-Leu, and Lys-Ala-Ile, were selected based on the published amino acid sequence of mouse cathepsin D. DNA aptamers against the tripeptides were isolated using the systematic evolution of ligands of exponential enrichment method. We have demonstrated that the aptamers specifically interacted with mouse cathepsin D using the structure-switch method. We further performed a proximity-dependent ligation assay to demonstrate that multiple aptamers could specifically detect the protein from cell extracts. In principle, one library containing 8000 aptamers should be enough to detect almost all proteins in the whole proteome in all organisms. This technology could be applied to generate a new generation of protein arrays.

Keywords: SELEX; Aptamer; Tripeptide; Protein ligand; Proximity-dependent ligation assay

Two-dimensional electrophoresis and mass spectrometry have been widely used for measuring and analyzing large numbers of proteins [1–5]. These technologies have limitations on their speed, sensitivity, throughput, and reproducibility [6]. Extensive studies have been conducted to develop protein arrays for protein expression and functional analysis [7–13]. The protein arrays are designed based on the physical and chemical interactions between known proteins and other proteins or nonprotein materials [14–16]. One of the most commonly used technologies was based on the antibody–antigen interaction [14,16]. For example, an antibody-based microarray containing 378 antibodies has been applied for protein expression profiling analysis [17]. Recently, RNA- and DNA-based aptamers were found to be as specific as antibodies for interacting with proteins [18–23]. These aptamers have been used in protein detection and protein arrays [24–34]. Small-molecule compounds that specifically interact with proteins were identified and used for protein array development [16,35–37]. However, unlike sequence-based DNA microarrays that cover most of the genes in the genome, these protein arrays cover only a very small fraction of proteins in the proteome. It has been estimated that posttranscriptional and posttranslational modifications may generate millions of proteins in a mammalian proteome [38–40]. Therefore, it is extremely difficult to identify and generate the specific molecular partners for every protein in the proteome. Indeed, there is no existing technology or protein array that can carry out protein expression analysis at the proteomic level. Nucleic acid aptamers that recognize

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and bind to proteins, neuropeptides, and even small molecules have been isolated [19,41–44]. Recently, many technologies using aptamers as ligands to recognize or detect proteins have been developed [45–48]. The proximity-dependent ligation assay [49–55], structure-switching aptamer technology [49–59] and other methods [48,60,61] have been successfully applied for protein detection [48,52,53,55,56,58,59,61–63]. Based on these methods, we have developed the novel DPAS[1] technology by which aptamers can detect proteins based on their amino acid sequences. We reasoned that, like antibodies, nucleicacid-based aptamers that have been generated against a small peptide should bind to the protein containing the peptide sequence on its surface. If more than one protein contains the peptide sequence, the aptamer should bind to all these proteins and therefore lack specificity. However, the combination of several aptamers that recognize and bind to their specific peptides at different parts of the protein should specifically recognize and detect the protein.

In this study, we have isolated aptamers against four tripeptides: GEL, DGI, KAI, and LAS. These four tripeptides are randomly distributed at mouse cathepsin D. Using the structure-switch method, we showed that the aptamers interact with mouse cathepsin D. Furthermore, the combination of four aptamers can specifically detect the protein using a multiple-aptamer-based proximity-dependent ligation assay (LA). Our results demonstrate that the DPAS technology may be applied to specifically detect the protein based on the amino acid sequence.

Materials and methods

Preparation of tripeptide-affinity column

The tripeptides Ac-LAS-amide, Ac-DGI-amide, Ac-GEL-amide, and Ac-KAI-amide were synthesized by New England Peptide, Inc. HiTrap NHS-activated columns were purchased from Amersham Pharmacia Biotech (Cat. No. 17-0716-01) and used to make the peptide affinity chromatography. The peptides were dissolved in standard coupling buffer (0.2 M $MaHCO_3$, 0.5 M NaCl, pH 8) to a final concentration of 0.5 mM. The column was washed with 6 ml of ice-cold 1 mM HCl at the flow rate of 1 ml/min. After column wash, 1 ml of the tripeptide solution was added and incubated at 25 °C for 30 min. The column was washed three times with 2 ml of 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3, and another three times with 0.1 M acetate, 0.5 M NaCl, pH 4. The wash procedure was repeated two more times. Finally, 2 ml of buffer A containing 20 mM Tris–HCl, pH 7.3, 140 mM NaCl, 5 mM KCl, 5 mM MgCl, 1 mM $CaCl_2$, and 0.02% Triton X-100 was added onto the column. The column was stored at 4 °C before use.

DNA aptamer library

The DNA pool (APT1-L) containing 60 random nucleotides with the sequence of GCA GTC TCG TCG ACA CCC (N)60 GTG CTG GAT CCG ACG CAG, where N represents A, T, G, or C, was synthesized and purified by polyacrylamide gel electrophoresis (PAGE). Sense primer (APT1-5) GCA GTC TCG TCG ACA CCC, antisense primer (APT1-3) CTG CGT CGG ATC CAG CAC, and antisense primer of APT1-3 (APT1-3A) GTG CTG GAT CCG ACG CAG were synthesized and PAGE purified. PCR was carried out in 50 μl of solution containing 1 μl of 4.4 μM APT1-L, 1 μl of 10 μM APT1-5, 1 μl of 10 mM APT1-3, 2 μl of water, and 45 μl of PCR SuperMix (Life Technology 10790-020). The reaction was incubated at 94 °C for 5 min, followed by 22 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s and finally 72 °C for 7 min. The PCR product was purified using PCR purification kit (Qiagen, 28106).

Single-stranded DNA generation

The purified PCR product was used as the template for the generation of single-stranded DNA. PCR was carried out in a total volume of 50 μl with 3 μl of the PCR product, 1 μl of 10 μM APT1-5, 1 μl of 10 μM APT1-3A for inactivating the remaining APT1-3 primer, and 45 μl of PCR SuperMix (Life Technology, 10790-020). The reaction was incubated at 94 °C for 5 min followed by repeated 22 cycles with 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and finally 72 °C for 7 min. For each tripeptide affinity column, PCR products from six tubes (300 μl) were pooled together into a 1.5 ml tube, 30 μl of 3 M Na acetate, 0.75 ml of 100% ethanol was added, and the tube was centrifuged to precipitate the PCR product. The single-stranded DNA was dissolved in 1 ml of buffer A, passed through a 0.2-μm filter, incubated at 70 °C for 10 min and at room temperature for 30 min, and then put on ice. The samples were ready to load onto the HiTrap NHS-activated tripeptide affinity column.

In vitro selection

The HiTrap NHS-activated tripeptide columns were washed three times with 2 ml of buffer A. The single-stranded DNA samples were loaded onto the columns. After 30 min at room temperature, the columns were washed one time with 11 ml of buffer A. DNA samples were eluted with three loadings of 0.8 ml of 0.5 mM tripeptide in buffer A. The samples were collected in six tubes, each tube contained about 400 μl of the eluted sample. DNA sample was precipitated by adding 1 ml of 100% ethanol. The precipitated DNA was dissolved in 6 μl of

[1] *Abbreviations used:* DPAS, detection of proteins based on amino acid sequences; GEL, Gly-Glu-Leu; DGI, Asp-Gly-Ile; KAI, Lys-Ala-Ile; LAS, Leu-Ala-Ser; NHS, *N*-hydroxy succinimidyl; Cat D, cathepsin D; SS, single-stranded; PMSF, phenylmethylsulfonyl fluoride; MAP, modified aptamer; LA, ligation assay; BSA, bovine serum albumin.

H_2O; 3 μl of the DNA sample was used for PCR containing 1 μl each of APT1-5 and APT1-3 primers and 45 μl of the SuperMix. The PCR was carried out with 22 cycles as described above. The PCR products from the six tubes were combined and purified by PCR purification kit; 3 μl of the PCR sample was used for the single-stranded PCR. The PCR condition was the same as above using primers Apt1-5 and Apt1-3A. The single-stranded PCR products were pooled, ethanol-precipitated, and used for the second round selection. This in vitro selection was repeated 12 times and the final PCR product was cloned into a TA vector and sequenced.

Binding assay

One microliter of the affinity-purified DNA aptamers was labeled with [^{32}P]ATP by single-stranded PCR using primer Apt1-5 for five cycles. The samples were purified using the PCR purification kit and counted. Ten thousand cpm samples were loaded onto the tripeptide affinity column. After 30 min incubation at room temperature, the columns were washed with 11 ml of buffer A. The remaining bound DNA was eluted with three loadings of 1 ml of 3 mM tripeptide in the same buffer. Each fraction was analyzed with Cerenkov counting. The number of counts from the eluted fractions was divided by the total counts to give the fraction of bound DNA.

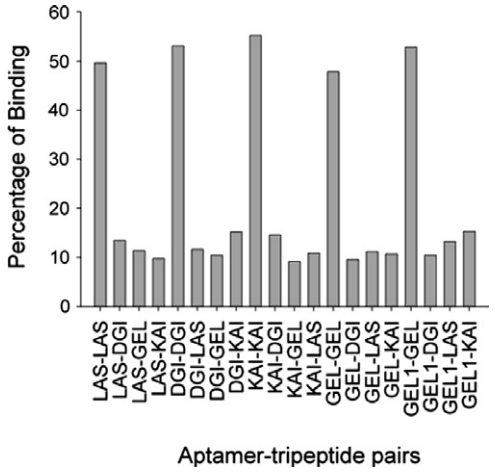

Fig. 1. Binding of the aptamers to different tripeptide affinity columns. Aptamers targeted to different tripeptides were analyzed by binding assay using tripeptide affinity columns. Radiolabeled DNA aptamers were incubated with tripeptides in the affinity columns, washed, and eluted and the percentage of bound DNA aptamers was determined.

Isolation of DNA aptamers against four tripeptides

We randomly selected four tripeptides, GEL, DGI, KAI, and LAS, from mouse Cat D protein for the isolation of specific aptamers. The tripeptides were synthesized and coupled to HiTrap NHS-activated column. The starting DNA pool was a mixture of 96-mer single-stranded DNA molecules containing randomized 60-nucleotide inserts. The ssDNA molecules that bound to the tripeptides were eluted and amplified by PCR. The PCR products were further selected using the tripeptide affinity columns for sublibrary generation. The selection procedure was repeated 12 times and the final products were cloned and sequenced. Ten aptamers were selected for each tripeptide and used for the binding assay. Four aptamers with high binding ability (Fig. 1), GEL-aptmer, KAI-aptmer, DGI-aptamer, and LAS-aptamer, were selected for the protein detection experiments (Tables 1 and 2).

Generation and purification of recombinant mouse Cat D and Escherichia coli Dahp proteins

Mouse Cat D was cloned from the mouse brain cDNA (Invitrogen) and Dahp was cloned from *E. coli* genomic DNA using PCR. The primers used for mouse Cat D were 5′-CCT GAA TTC ATG AAG ACT CCC GGC GT-3′ and 5′-ATC AAG CTT GAG TAC GAC AGC ATT GGC-3′; those for Dahp were 5′-GAA TTC ATG AAT TAT CAG AAC GAC GAT TTA CG-3′ and 5′-AAG CTT CCC GCG ACG CGC TTT TAC T-3′. The coding regions of Cat D and Dahp were inserted into pET 24a(+) vector (Novagen) and grown in *E. coli* BL21(DE3) (Novagen). Cells from 100 ml of broth were collected after incubation with 1 mM isopropyl β-D-thiogalactoside for

Table 1
Sequences of aptamers, FDNA, and QDNA for structure-swiching assay

Name	Sequence
MAP-KAI	***CCTGCCACGCTCCGCCCTGC***TCACTGGCGCAGCGGGTGGAGTGTTAAGATGAATTGCGGTGTGGGCCGGCCTCTATTGGC
MAP-GEL	*CCTGCCACGCTCCGCCCTGC***TCACTGGCGAAGCGGG**CTGAAGTGCACACAGCTGGAGGAGTATTGTTGGGTGCTC
MAP-LAS	***CCTGCCACGCTCCGCCCTGC***TCACTGACGAAGTGGGTGTATAGCGAATAATCATTAAGAAAGGGCGCTGTGTTGTG
	CCTGCCACGCTCCGCCCTGCTCAGTGAGCCTAAAATATTGCTTAGTAAGGGTGGTCTGGCTCCGAGAGGGGT
FDNA	FAM-***GCAGGGCGGAGCGTGGCAGG***
QDNA	**CCCGCTGCGCCAGTG**–DABCYL
DGI-QDNA	**ATTTTAGGCTCAGTG**–DABCYL
LAS-QDNA	**CCCACTTCGTCAGTG**–DABCYL

The sequences of aptamers are underlined, FDNA and its antisense sequences are indicated in italics, and the QDNA and its antisence sequences are shown in boldface.

Table 2
Sequences of aptamers, connectors, and primers for proximity-dependent ligation assay

Name	Sequence
LA-GEL	**ACTTCAGCCCTTTTTTTTAATCACTTAT**GCGAAGC**GGGCTGAAGT**GCACAC**AGCTGGA GGAG**TATTGTTGGGTGCTC**TTTC**TCCTCCAGC**
LA-KAI	**TTAACACTCC**TTTTTTTTATCTT**GCGCAGCGGGT GGAGTGTTAA**GATGAATT**GCGGTGTGGG** CCGGCCTCTATTGGC**TTTCCCACACCGC**
LA-LAS	**CGCTATACAC**TTTTTTATCACTTAT**ACGAAGTGG GGTGTATAGCG**AATAATCA**TTAAGAAAGG** GCGCTGTGTTGTG**TTTTTCCTTTCTTAA**
LA-DGI	**TAAGCAATAT**TTTATCACTTATCCAT**AGCCTAAAATATTGCTTA**GTAAGGGT**GGTCTGGCTC**CCG AGAGGGGT**TGAATTCT**GAGCCAGACC**
LA-GEL1	**AACATCTACG**TTTTTTTTAATCACTTAT**AGAGGGC CGTAGATGTT**ATACTGT**GGGTAGTATA**GGCTTGGT TTTTATACTACCC**
Connector1	5′-AAAGGGCTGAAGTGGTCTGGCTCTTT-3′
Connector2	5′-AAAGGAGTGTTAAGCTGGAGGAGTTT-3′
Connector3	5′-AAAGTGTATAGCGGCGGTGTGGGTTT-3′
Connector4	5′-AAAATATTGCTTATTAAGAAAGGTTT-3′
Primer1	5′-CGAAGCGGGCTGAAGTGCA-3′
Primer2	5′-TCTTAACACTCCACCCGCT-3′
Connector5	5′-TTTCGTAGATGTTGGTCTGGCTCTTT-3′
Connector6	5′-TTTGGGCTGAAGTGGGTAGTATATTT-3′
Primer2f	5′-GGAGTGTTAAGCTGGAGGAG-3′
Primer3f	5′-GTGTATAGCGGCGGTGTGGG-3′
Primer4f	5′-ATATTGCTTATTAAGAAAGG-3′
Primer5r	5′-GAGCCAGACCAACATCTACG-3′
Primer6r	5′-TATACTACCCACTTCAGCCC-3′

The sequences of aptamers are underlined and the arms and their antisense sequences in the aptamers are indicated in boldface.

4 h at 37 °C. The Dahp protein was purified as described by Ni-nitrilotriacetic acid manual (Novagen).

The recombinant mouse Cat D was purified using a precipitation method as described [64]. Briefly, cells were incubated in 30 ml Phosphate-buffered saline containing 1 mM PMSF and 1 g/L lysozyme for 20 min on ice. After the incubation, 0.3 ml Triton X-100 was added and the cell suspension was incubated on ice for 10 min. The suspension was sonicated for 30 s and centrifuged at 15,000 rpm using the Beckman JA-14 rotor for 15 min at 4 °C. The pellet was resuspended in 20 ml of 10 mM EDTA solution, pH 8.0, sonicated for 30 s and then collected by centrifugation in a Beckman JA-14 rotor at 5000 rpm for 15 min at 4 °C. The EDTA precipitation procedure was repeated three times. The pellet was then resuspended in 15 ml of 2 mM EDTA solution, pH 8.0. The suspension was sonicated until the sample became white. Then 15 ml of cold 40 mM NaOH was added to the sample which was sonicated again until the sample turned to clear solution. Finally, 7.5 ml of 40% glycerol solution containing 5 mM PMSF and 5% leupeptin was added and the sample was stored at −70 °C.

Structure-switching assay

The structure-switching assay was performed as previously reported [59]. Briefly, the DNA aptamer was modified with an addition of a short oligonucleotide sequence at the 5′ end (MAP). Fluorescent-group-labeled oligonucleotide (FDNA) and quench-group-labeled oligonucleotide (QDNA) were synthesized (Sangon). The sequences are listed in Table 1. The assay was carried out with 160 nM MAP, 320 nM FDNA, 480 nM QDNA, and different concentrations of proteins in 20 μl of buffer A. The reaction was incubated at 37 °C for 60 min. DNA engine OPTICON2 continuous fluorescence detector (MJ Research) was used to measure the fluorescence signals generated by the interaction between the aptamers and the proteins.

Proximity-dependent ligation assay

The aptamers (LA-GEL, LA-KAI, LA-LAS, and LA-DGI) for the ligation assay were synthesized and the sequences are listed in Table 2. The connectors and primers were connector1, connector2, connector3, connector4, primer1, and primer2 (Table 2). In the experiment with five aptamers (LA-GEL, LA-KAI, LA-LAS, LA-DGI, and LA-GEL1), the connectors and primers were connector5, connector6, primer2f, primer3f, primer4f, primer5r, and primer6r (Table 2). The mouse Cat D protein, truncated Cat D protein, *E. coli* lysis and BSA were diluted with 1% BSA. One microliter of aptamer at the concentration of 10 pM was incubated with 5.0 μl of proteins at room temperature for 1 h. Seven microliters of distilled water, 1.4 μl of 5× T4 DNA ligase buffer, 0.2 μl of T4 DNA ligase (Invitrogen), 0.4 μl of connectors (25 μM), and 1.0 μl of the preincubated protein mixture were added to a tube, mixed, and incubated at room temperature for 5–7 h. After the ligation, 1 μl of each sample was subjected to hyperbranched rolling circle amplification [65–67], in a total volume of 10 μl in the presence of 50 mM Tris–HCl,

pH 7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM dithiothreitol, 200 μg/ml BSA, 10 U of phi29 DNA polymerase (New England Biolabs), 1.0 μM each primer, and 0.5 mM dNTPs. The reaction was incubated for 8–24 h at 30 °C followed by 10 min at 65 °C to inactivate the polymerase. The amplified products were detected with 1% agarose gel electrophoresis.

Database search

We have conducted a pattern search in the PIR database (http://pir.georgetown.edu/pirwww/search/pattern.shtml) [68–71] to identify proteins containing the tripeptides. We initially searched for proteins containing the sequence LASX(1,300)DGIX(1,300)GELX(1,300)KAI, where X(1,300) indicates that there are 1–300 amino acids between the two tripeptides. We then used permutations of tripeptides to conduct the pattern search one by one (Table 3). The searches covered the summed occurrences of all tripeptides. We searched the PIR NREF database to identify proteins containing either three (GEL, DGI and KAI) or (GEL, DGI, KAI, and LAS) four tripeptide sequences. We found more than 1000 proteins that contain three of these tripeptide sequences in humans. There were only 2, 17, and 35 proteins that contained all four tripeptides in *E. coli*, mouse, and human, respectively (Table 3).

Table 3
Number of proteins containing four tripeptides in *E. coli*, mouse, and human

Permutation of tripeptides	*E. coli*	*Mus*	*Homo*
LASX(1,300)DGIX(1,300)GELX(1,300)KAI	1	7	3
LASX(1,300)DGIX(1,300)KAIX(1,300)GEL	0	1	0
LASX(1,300)GELX(1,300)DGIX(1,300)KAI	0	0	0
LASX(1,300)GELX(1,300)KAIX(1,300)DGI	0	0	2
LASX(1,300)KAIX(1,300)GELX(1,300)DGI	1	0	5
LASX(1,300)KAIX(1,300)DGIX(1,300)GEL	0	0	3
DGIX(1,300)LASX(1,300)GELX(1,300)KAI	0	0	2
DGIX(1,300)LASX(1,300)KAIX(1,300)GEL	0	0	0
DGIX(1,300)GELX(1,300)LASX(1,300)KAI	0	0	0
DGIX(1,300)GELX(1,300)KAIX(1,300)LAS	0	1	2
DGIX(1,300)KAIX(1,300)GELX(1,300)LAS	0	0	3
DGIX(1,300)KAIX(1,300)LASX(1,300)GEL	0	0	0
GELX(1,300)DGIX(1,300)LASX(1,300)KAI	0	0	3
GELX(1,300)DGIX(1,300)KAIX(1,300)LAS	0	0	0
GELX(1,300)LASX(1,300)DGIX(1,300)KAI	0	0	1
GELX(1,300)LASX(1,300)KAIX(1,300)DGI	0	0	2
GELX(1,300)KAIX(1,300)LASX(1,300)DGI	0	2	5
GELX(1,300)KAIX(1,300)DGIX(1,300)LAS	0	4	3
KAIX(1,300)DGIX(1,300)GELX(1,300)LAS	0	0	0
KAIX(1,300)DGIX(1,300)LASX(1,300)GEL	0	0	0
KAIX(1,300)GELX(1,300)DGIX(1,300)LAS	0	0	0
KAIX(1,300)GELX(1,300)LASX(1,300)DGI	0	0	0
KAIX(1,300)LASX(1,300)GELX(1,300)DGI	0	0	1
KAIX(1,300)LASX(1,300)DGIX(1,300)GEL	0	2	0
Total number	2	17	35

All permutations of four tripeptides are listed and used for search in the PIR-NREF database (http://pir.georgetown.edu/pirwww/); 1–300 random amino acids are inserted between each pair of tripeptides.

Results

Detection of aptamer–protein interaction

We have used structure-switching technology [59] to detect the aptamer–protein interactions. The advantage in using this method is that the aptamers can be labeled with fluorescent groups without affecting the secondary structure of the aptamer [58,59]. Furthermore, the fluorescence signals can be easily detected as soon as the aptamers interact with proteins [58,59]. The modified aptamers contain about 30 nucleoside bases at the 5′ ends followed by the full-length aptamer sequences (Table 1). Two other oligonucleotides were used for this assay (Table 1), one labeled with a fluorophore (FDNA) and another labeled with a quencher (QDNA) [58,59]. The FDNA was designed to anneal to the 5′ end of MAP while most of the QDNA should interact with the motif of aptamer. This design should bring the fluorophore and the quencher together and therefore no fluorescence signals should be generated [58,59]. When the aptamer interacts with the target protein, the QDNA is disassociated from the aptamer and the fluorescence signals can be generated from the FDNA [58,59]. We added different concentrations of partially purified recombinant mouse Cat D protein into the system. Our results demonstrated that addition of the protein resulted in the elevation of fluorescence signals in a dose-response manner (Fig. 2). BSA, a protein without the tripeptide sequences, was used as a control. We found that addition of BSA did not induce any fluorescence signal even at very high concentrations (Fig. 2). This result showed that there was no interaction between the BSA and the aptamers. To further test the specificity of the aptamer–protein interaction, we used *E. coli* Dahp protein, which contains two KAI sequences for the structure-switching experiment. We found that Dahp induced the fluorescence emission when MAP-KAI was present in the assay, indicating an interaction between Dahp and KAI-specific aptamer (Fig. 2). As expected, higher concentrations of Dahp protein generated more fluorescence signals. Surprisingly, the addition of BSA at high concentrations slightly increased the signal. It is possible that BSA in the solution might affect the stability of the structure-switching aptamers and therefore slightly increased the signals. Taken together, these results suggest that aptamers targeting tripepetides can bind to proteins containing the tripeptide sequences and that the interactions between the aptamers and the proteins are specific.

Detection of cat D protein based on the amino acid sequence

We next tested whether the combination of several aptamers could specifically detect the mouse Cat D protein. Proximity-dependent ligation assay [49,53,55] was used for this purpose (Fig. 3). In this assay, much effort has been expended to optimize the ligation system to reduce background ligation [55]. In our experiment, two arms with 10

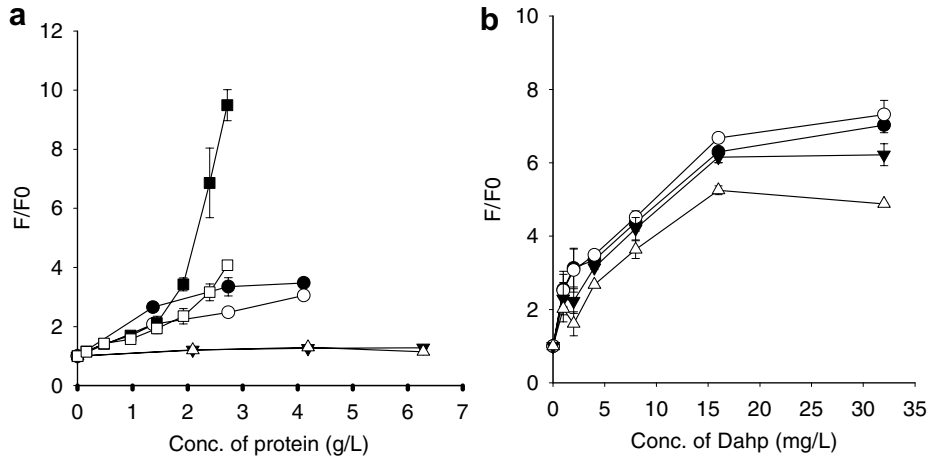

Fig. 2. Interaction between aptamers and proteins by structure-switching methods. (a) Structure-switching signaling aptamer method was used to detect the interaction between the mouse Cat D protein and the aptamers. The DGI-aptamer, KAI-aptamer, GEL-aptamer, or LAS-aptamer (□, LAS-aptamer and Cat D, ■, DGI-aptamer and Cat D; ○, KAI-aptamer and Cat D; ●, GEL-aptamer and Cat D; ▼, KAI-aptamer and BSA; △, GEL-aptamer and BSA) were used for the assay. Different concentrations of Cat D (0, 1.4, 2.7, and 4.1 g/L for KAI and GEL; 0, 0.16, 0.48, 0.96, 1.44, 1.92, 2.4, and 2.72 g/L for DGI and LAS) and BSA (0, 2.0, 4.2, and 6.3 g/L) proteins were added. The concentrations of MAP, FDNA, and QDNA were 160, 320, and 480 nM, respectively. The fluorescence intensity of each sample was normalized with F/F0, where F is the fluorescence intensity of each sample and F0 is the initial signal in the absence of target proteins. (b) KAI-aptamer was used in the assay. Dahp protein concentrations were 0, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 mg/L. BSA concentrations were 0 g/L (△), 0.4 g/L (▼), 2.0 g/L (○), and 4.0 g/L (●). The fluorescence intensity of each sample was normalized with F/F0, where F is the fluorescence intensity of each sample and F0 is the initial signal in the absence of protein targets.

nucleotides were added to both ends of the aptamers in such a way that the arms could anneal to the motif of the aptamer. This intramolecular interaction could block the annealing reaction between the connectors and the aptamers, therefore reducing the background of the proximity-dependent ligation [55]. The target protein may interact with the aptamers, leading to the conformation change of the aptamers and resulting in the release of the arms. The connectors should then be able to anneal to the aptamers for the proximity-dependent ligation. We have added four different aptamers to the reaction system that contained partially purified recombinant mouse Cat D protein. In the reaction system, the interaction between aptamers and Cat D protein may lead to the release of the arms and the formation of a ring. The single-stranded DNA ring could then be rolling circle amplified by Phi29 DNA polymerase [72,73] (Fig. 3). The amplified products were detected in 1% agarose gel. Our results showed that the protein could be detected in a dose response manner (Fig. 4a). Importantly, the technology could detect the protein at a concentration as low as 100 amol. To test the specificity of the aptamer–protein interaction, we used a truncated mouse Cat D protein lacking the LAS tripeptide at the N terminus. Therefore, these four aptamers could not form a ring for the rolling circle amplification. Indeed, our result showed that no amplified product could be detected even when 10 nmol of the truncated protein was used in the assay system (Fig. 4a).

We further tested whether the recombinant Cat D protein could be detected in a cell extract that contained a large number of other proteins using the proximity-dependent ligation assay. To increase the specificity, we used five aptamers for the assay (Table 2). Since Cat D protein contains two GEL tripeptide sequences, two GEL, one LAS, one DGI, and one KAI aptamers were added to the reaction containing the Cat-D-vector-transformed *E. coli* extract. Mock-vector transformed *E. coli* extract was included as the control. Our results demonstrated that these five aptamers could effectively detect Cat D protein with high specificity and sensitivity, while no signal was detected from the mock-transformed cell extract (Fig. 4b). In conclusion, the novel DPAS technology has been successfully used to detect the mouse Cat D protein based on its amino acid sequence in a cell extract.

Discussion

The DPAS technology has been designed and developed based on two important assumptions. First, a nucleic acid aptamer that specifically binds to a tripeptide should be able to recognize and interact with the tripeptide in the protein that contains the accessible tripeptides on the surface of the protein and the tripeptide should retain the same three-dimensional conformation. Second, the combination of several such aptamers can specifically detect and capture the particular protein that contains these tripeptides. To perform a proof of principle experiment, we selected four tripeptides based on the amino acid sequence of mouse cathepsin D protein for the generation of specific DNA aptamers. It has been shown that the tripeptides adopt stable structures in water, which at least to some extent reflects the intrinsic structural property of the respective amino acids in proteins [74,75]. Aptamers targeted to short peptides should dominate the structure of the complex [76]. Therefore, we hypothesized that DNA aptamers targeting to tripeptides should be able to interact with proteins con-

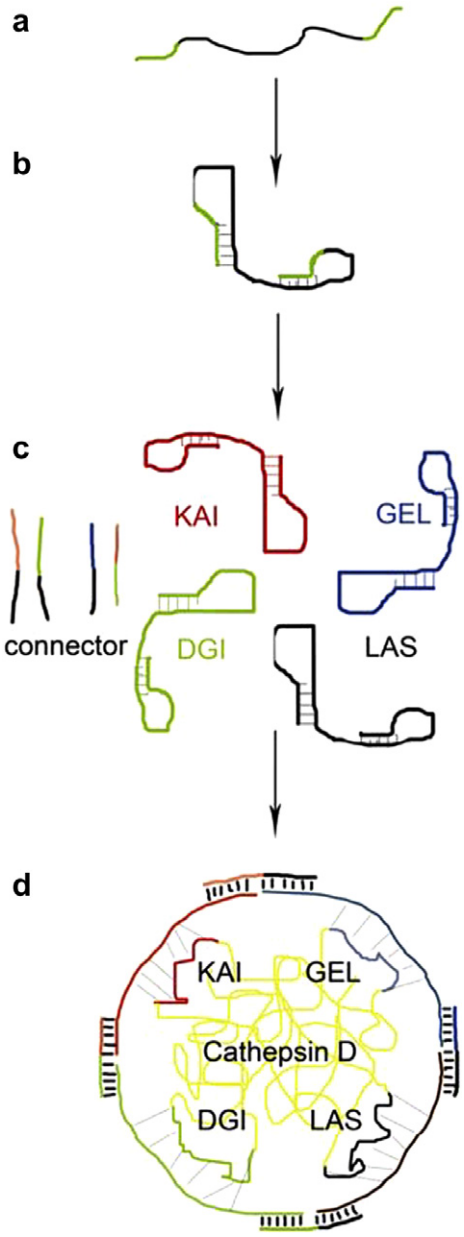

Fig. 3. Schematic representation of the multiple-aptamers-based proximity-dependent ligation assay. LA-GEL-aptamer, LA-KAI-aptamer, LA-LAS-aptamer, and LA-DGI-aptamer were generated by adding arms at each end of the corresponding aptamers (a, b). The arms anneal to the aptamer itself which could reduce the background ligation and increase the specificity of interaction with targets (b, c). When the Cat D protein was added to the system, the arms of the four aptamers could be ligated together for rolling circle amplification (d). Dotted lines represent the interactions between the tripeptides and their aptamer ligands. Short lines with two different colors are connectors for the ligation of the aptamers (c, d).

taining the tripeptide sequences. The structure-switching method was applied to test this hypothesis. Our results demonstrated that DNA aptamers targeting to tripepetides could interact with the proteins containing the tripeptide sequences. Furthermore, the proximity-dependent ligation assay demonstrated that multiple aptamers could specifically detect the protein based on the amino acid sequence.

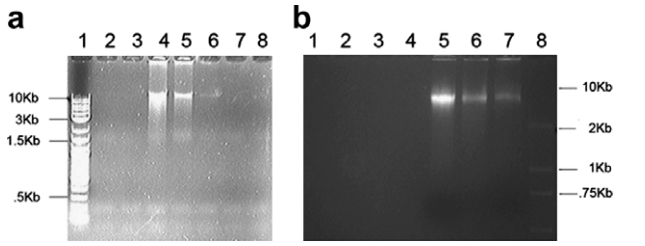

Fig. 4. Detection of Cat D protein by proximity-dependent multiple-aptamers ligation assay. (a) Four-aptamers-based proximity-dependent ligation assay. lane 1, molecular weight marker; lane 2, 10.0 nmol truncated Cat D; lane 3, 1.0 nmol truncated Cat D; lane 4, 10.0 fmol Cat D, lane 5,1.0 fmol Cat D; lane 6, 100.0 amol Cat D; lane 7, 1.0 amol Cat D; lane 8, 140 nmol BSA. (b) Five-aptamers-based proximity-dependent ligation assay. Lane 1, 140 nmol BSA; lane 2, 72.5 ng mock-transformed *E. coli* protein extract; lane 3, 0.7 ng mock-transformed *E. coli* protein extract; lane 4, 7.25 pg mock-transformed *E. coli* protein extract; lane 5, 72.5 ng Cat-D-vector-transformed *E. coli* protein extract; lane 6, 0.7 ng Cat-D-vector transformed *E. coli* protein extract; lane 7, 7.25 pg Cat-D-vector transformed *E. coli* protein extract; lane 8, molecular weight marker.

Aptamers against tetrapeptides, pentapeptides or hexapeptides could be used for the development of DPAS technology. We have chosen tripeptides for the proof of principle experiment mainly on a consideration of the size of the ligand library. The maximum number of aptamers for tripeptides needed to cover all possible combinations of 20 amino acids is 8000 ($20 \times 20 \times 20$), while the number reaches 160,000 for tetrapeptides, 20 times more for pentapeptides, and so on. Our database search results demonstrated that there were only 2, 17, and 35 proteins that contain these four tripeptide sequences in *E. coli*, mouse, and human, respectively (Table 3). Furthermore, systematic analysis of protein sequences using bioinformatics tools may significantly reduce the number of aptamers needed to recognize and detect all the proteins in the proteomes. These results suggest that, if we select the tripeptide aptamers based on protein sequence analysis, we should be able to use four aptamers, or at most five, to detect a specific protein.

It is important to note that the DPAS technology is to detect proteins based on amino acid sequences. Therefore, the proteins need to be denatured and the tripeptide sequences should be exposed to the solution for aptamer binding. However, this technology may also be applied to detect undenatured native proteins if the aptamers recognize the amino acid sequences on the surfaces of the proteins. Aptamers for those peptides containing the polar amino acids, such as Lys, Arg, Asp, Asn, Gln, and Glu, are likely exposed on the surface of soluble protein. Recently, a number of methods, including antibody-based protein array [77], peptide-based protein array [78,79], photo-atpamer-based protein array [34,80–82], proximity-dependent ligation assay [55], aptamer-based exonuclease protection assay [47], and structure-switching aptamer technology [59], have been developed for protein detection and expression analysis. These methods cannot be applied to recognize and detect protein targets based on their

amino acid sequence information. It is not possible to conduct proteome wide analysis due to the unavailability of ligands for most proteins in the proteome. However, the DPAS technology appears to recognize proteins based on the sequence information. In principle, a ligand library containing 8000 aptamers could recognize and detect all the proteins in the proteome for all organisms.

In summary, the novel DPAS technology was able to detect specific proteins based on their amino acid sequences. We found that DNA aptamers against tripeptides isolated in vitro could recognize and interact with intrinsic motifs containing the tripeptide sequences. Moreover, the combination of four or five aptamers could specifically detect the protein based on the amino acid sequence. In the development of the DPAS technology, we have modified the proximity-dependent ligation assay [55] by using multiple aptamers to form a ring and then conduct rolling circle amplification [72,73,83,84]. We further modified the method to reduce the background ligation with arms annealing to the aptamers. These modifications made the DPAS technology more flexible and sensitive and enabled the multiple-tripeptide-sequence-based detection of protein [85–87]. The DPAS technology could be applied to the development of a new generation of amino-acid-sequence-based protein arrays.

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