Nucleic Acids Research, 2003, Vol. 31, No. 19 ***e118***
DOI: 10.1093/nar/gng119

DNA display for *in vitro* selection of diverse peptide libraries

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Received July 16, 2003; Revised and Accepted August 16, 2003

ABSTRACT

We describe the use of a DNA display system for *in vitro* selection of peptide ligands from a large library of peptides displayed on their encoding DNAs. The method permits completely *in vitro* construction of a DNA-tagged peptide library by using a wheat germ *in vitro* transcription/translation system compartmentalized in water-in-oil emulsions. Starting with a library of 10^9–10^{10} random decapeptides, 21 different peptide ligands were isolated for monoclonal antibody anti-FLAG M2. DNA display selected more diverse peptides with a DYKXXD consensus motif than previously reported phage display systems. Binding and recovery rates of three peptides were significantly higher than those of the original FLAG peptide, implying that these peptides would be superior to the FLAG peptide for purification of tagged proteins. The simplicity of DNA display enables two selection rounds per day to be conducted. Further, DNA display can overcome the limitations of previous display technologies by avoiding the use of bacterial cells and RNA tags. Thus, DNA display is expected to be useful for rapid screening of a wide variety of peptide ligands for corresponding receptors.

INTRODUCTION

In vitro selection and directed evolution of proteins can be achieved by linking phenotype (polypeptides) and genotype (nucleic acids) (1–3). For example, phage display allows physical association of the peptide phenotype displayed on the surface of phage particles with the encapsulated DNA genotype (4). However, the diversity and complexity of the phage libraries are limited by the cell transformation step. Ribosome display (5–7) and mRNA display (8,9) permit totally *in vitro* construction of larger libraries by linking mRNA with its nascent polypeptide. RNA is less stable than DNA, however. RNA can be replaced by cDNA in mRNA display systems (10,11), but screening of peptides or proteins from large libraries in such systems has not been reported. Thus, a completely *in vitro* system which links peptides to their corresponding DNAs offers several advantages over current methods in evolutionary biotechnology.

Linkage between mRNA and the nascent polypeptide can be accomplished on the ribosome (5–9). In contrast, DNA–peptide linkage requires compartments that contain the DNAs and the peptides they encode. While phage display utilizes *Escherichia coli* cells as the compartments, we employed *in vitro* compartmentalization in water-in-oil emulsions (12) to construct a totally *in vitro* DNA display system. *In vitro* compartmentalization was originally developed by Tawfik and Griffiths for the directed evolution of enzymes (12–14). We applied it to link peptides with their encoding DNAs in order to select peptide ligands (15). A streptavidin–biotin complex was used as a connector for the peptide–DNA linkage, and the method was named STABLE to indicate 'streptavidin–biotin linkage in emulsions' (15). However, the efficiency of affinity selection was very limited and, therefore, establishment of an efficient DNA display system required further improvements of our STABLE system. In this study, we have established a DNA display system and applied it to screen diverse peptide ligands for a monoclonal antibody from a random decapeptide library.

MATERIALS AND METHODS

DNA construction

The oligonucleotide sequences used in this study are listed in Table 1. A DNA fragment that contains an SP6 promoter, translational enhancer from tobacco mosaic virus (16) and a synthetic streptavidin gene of low GC content (54%) was constructed as follows. DNA fragments (Fragments 1–5) were assembled by overlap extension PCR with KOD Plus DNA polymerase (Toyobo) using PT7SP6 and Sta159R primers. The fragment was inserted into the BglII and XhoI sites of pT7-STA (15), yielding pSta4. A streptavidin-fused random decapeptide library was amplified from pSta4 with T7F and STA-Random primers and re-amplified with biotin-labeled T7F and T7R primers. PCR products were purified with a QIAquick kit (Qiagen). An expressible DNA fragment encoding a streptavidin–FLAG fusion protein was prepared

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Table 1. Oligonucleotide sequences

Oligo	Sequence
Fragment 1	ATTTAGGTGACACTATAGAACAACAACAACAACAAACAACAACAAAATGGCTAGCATGACTGGTGGACAGCAAATGGGT
Fragment 2	TCGTAAGTACCGGTAAGAGCACCATCAGCGCCTGCGGTCACAATAAAGGTAGAGCCAAGTTGGTTGTACCAGGTGCCGGT GATGCCAGCTTGATCGCGACCCATTTGCTGTCCACCAGTCATGCTAGCCAT
Fragment 3	TGCTCTTACCGGTACTTACGAATCTGCTGTCGGTAACGCTGAATCTCGCTACGTCCTTACTGGTCGCTATGATTCTGCTCC AGCTACTGATGGTTCTGGCACTGCTCTTGGTTGGACTGTTGCTTGGAAGAAT
Fragment 4	CCAAGCGTTAGCTTCGGTGGTACCAGAGGTAAGAAGCCATTGGGTGTTAATGCGTGCTTCAGCGCCGCCGACGTATTGG CCAGACCAAGTGGTTGCAGAATGAGCGTTGCGGTAGTTATTCTTCCAAGCAACAGTCC
Fragment 5	CCACCGAAGCTAACGCTTGGAAATCTACTCTTGTCGGCCATGATACCTTCACCAAAGTGAAACCATCTGCTGCTTCTAT CGATGCAGCTAAGAAAGCTGGCGTCAACAATGGCAACCCGCTGGACGCTGTTCAGCAG
PT7SP6	ATCAGATCTATCCCGCGAAATTAATACGACTCACTATAGGGATTTAGGTGACACTATAG
Sta159R	TTTCTCGAGTCACTGCTGAACAGCGTCCAG
T7F	CGGCATATGATCCCGCGAAATTAATACG
T7R	GCTAGTTATTGCTCAGCGG
STA-Random	GCTAGTTATTGCTCAGCGGTTAVNNVNNVNNVNNVNNVNNVNNVNNVNNVNNCTGCTGAACAGCGTCCAGCG
STA-FLAG	GCTAGTTATTGCTCAGCGGTTACTTGTCGTCATCGTCCTTGTAGTCGGATCCCTGCTGAACAGCGTCCAGCG
T7tagR	ACCCATTTGCTGTCCACCAGTCATG

Oligonucleotide sequences are indicated in the 5′→3′ direction. N = A, C, G or T; V = A, C or G. T7F double-labeled with biotin and fluorescein and T7R labeled with biotin were used for preparation of labeled DNAs.

similarly using a STA-FLAG primer to serve as a positive control. All DNA sequences were confirmed with a CEQ2000 sequencer (Beckman Coulter).

Affinity selection

An *in vitro* transcription/translation reaction in water-in-oil emulsions was performed as previously described (12,15) with modifications. Fifty microliters of a reaction mixture of the TNT SP6 coupled wheat germ extract system (Promega) was added to a stirred oil phase containing 44 µl of Span 85 (Nacalai Tesque) and 6 µl of Tween 20 (Sigma) in 900 µl of mineral oil (Nacalai Tesque) at 4°C. The DNA concentration was 50 pM in each round of selection except for the first round, in which it was 200 pM, and the fifth round, in which it was 10 pM. After a 90 min reaction at 30°C, the emulsions were broken with 200 µl of ice-cold quenching buffer [TBST, 200 nM biotin, 1% protease inhibitor cocktail (Sigma) and 1% BSA] and 1 ml of ice-cold water-saturated diisopropyl ether. The mixture was inverted 20 times and centrifuged at 12 000 r.p.m. for 5 min at 4°C. The water phase was filtered through an Ultrafree-MC (0.1 µm) (Millipore) and subjected to vacuum for 15 min to remove residual diisopropyl ether. Then, 160 µl of this sample was added to 25 µl of anti-FLAG M2–agarose beads (Sigma), which had been pre-equilibrated with TBST, and mixed on a rotator for 1 h at 4°C. The beads were washed extensively with TBST, 50 µl of elution buffer [1 mg/ml FLAG peptide (Sigma), 50 mM Tris–HCl, 50 mM NaCl, pH 7.6] was added and rotation was continued for 10 min at 25°C. PCR reactions containing 1/10 vol of the eluate were performed with KOD Plus DNA polymerase using biotinylated T7F and T7R primers. The PCR program was 25–30 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 20 s and extension at 72°C for 50 s. The DNA products were used for the next round of selection. To identify selected peptides, DNA was amplified with non-labeled primers, cloned and sequenced.

Dot blot analysis

Total DNA from each round of selection was transcribed and translated *in vitro* and the products were spotted in triplicate onto a nitrocellulose membrane (Optitran BA-S 83; Schleicher & Scheull) and dried. Products were detected with a 1:5000 dilution of anti-T7·tag (Novagen) or a 1:1000 dilution of anti-FLAG M2 antibody (Sigma) and a 1:2500 dilution of horseradish peroxidase-conjugated secondary antibody (Chemicon). The intensity of each spot was normalized to that of anti-T7·tag.

Protein microarray

Protein microarrays were prepared and probed as described (17). Streptavidin-fused peptides were synthesized with the TNT SP6 wheat germ extract system (Promega) from randomly chosen DNA clones and printed onto biotin-coated slides (18). Peptides were detected with anti-T7·tag or anti-FLAG M2 followed by Cy3-conjugated secondary antibody (Chemicon).

Pull-down assay

DNA–peptide conjugates for selected clones were prepared separately with 10 nM DNA, incubated with anti-FLAG M2–agarose beads, washed and eluted as described above. Input and eluate fractions were diluted 1:2000 and analyzed by quantitative real-time PCR with T7F and T7tagR primers using a LightCycler (Roche) in at least two independent experiments.

Surface plasmon resonance spectroscopy

Dissociation constants were measured with a Biacore 3000 instrument (Biacore) according to the manufacturer's instructions. Anti-FLAG M2 antibody (~7000 RU) was immobilized on a CM5 sensor chip (Biacore) by amine coupling. FLAG peptide and other synthetic peptides (Qiagen) were dissolved in HBS-EP buffer (Biacore), diluted to various concentrations (0.625–200 µM) and used as analytes. Data were analyzed

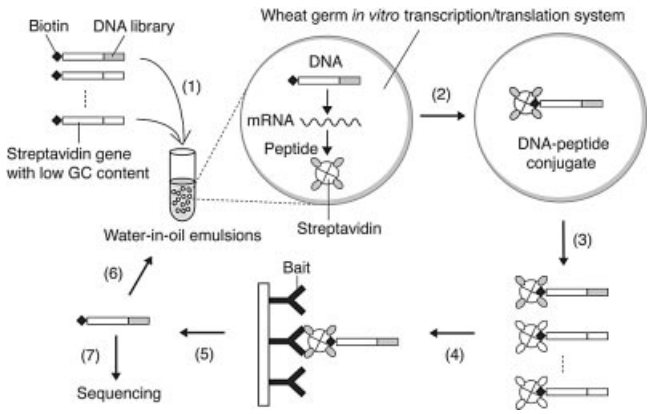

Figure 1. Schematic representation of the DNA display selection procedure. (1) A DNA library encoding streptavidin-fused peptides is labeled with biotin and compartmentalized in water-in-oil emulsions containing an *in vitro* transcription/translation system. (2) In each compartment, streptavidin-fused peptides are synthesized and attached to the template DNA via biotin labels. (3) DNA–peptide conjugates are recovered from the emulsion and (4) subjected to affinity selection on an immobilized bait. (5) After washing and elution, the DNA portion of the bound molecules is amplified by PCR. (6) DNA is subjected to the next round of selection or (7) identified by sequencing.

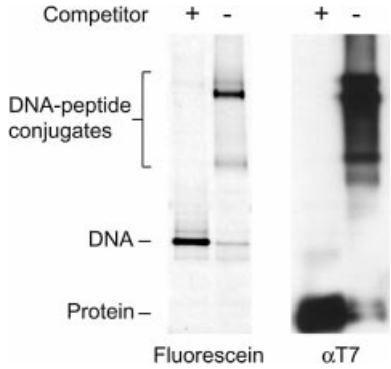

Figure 2. Formation of DNA–peptide conjugates. *In vitro* transcription/translation was performed with 10 nM DNA labeled with biotin and fluorescein in the presence (+) or absence (–) of 0.1 mM free biotin competitor. Each product was resolved in an 8% SDS–PAGE gel and analyzed with fluorescein (left) or by western blotting using an anti-T7·tag (right). DNA–peptide conjugates had lower mobilities than free DNA or protein. Multiple bands of conjugates originate from binding of different numbers of DNA molecules to a tetramerized streptavidin-fused peptide in non-emulsified reactions.

with the 'affinity analysis' model in the BIAevaluation software (Biacore).

RESULTS AND DISCUSSION

Establishment of a DNA display system

Formation of DNA–peptide conjugates is an essential step in DNA display (Fig. 1). In our previous study, these conjugates were formed very inefficiently. Only 1% of input DNA associated with peptides (15). This was probably due to low yields of streptavidin-fused peptides relative to the input

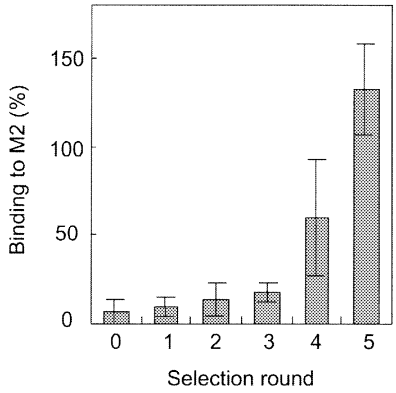

Figure 3. Fraction of random decapeptide library that bound to anti-FLAG M2 antibody and was eluted with FLAG peptide, at each round of selection. The intensity of the selected peptide mixture relative to the original FLAG peptide is indicated.

biotinylated DNA in an *E.coli* S30 *in vitro* transcription/translation system. In the present study, we improved the efficiency by using a wheat germ *in vitro* transcription/translation system. DNA–peptide conjugates were formed with an efficiency of >95% (Fig. 2).

The previous system was further improved in two respects. First, biotin molecules were added at both 5′-ends of each DNA fragment to display multiple peptides per DNA molecule. As streptavidin forms a tetramer, a mono-biotinylated DNA binds to a tetrameric streptavidin-fused protein. Thus four copies of peptides are displayed (Fig. 1). Having biotin on both ends of the DNA means that eight copies of peptide can be displayed per DNA molecule. Increased avidity of the di-biotinylated DNA significantly improved both the adsorption of DNA–peptide conjugates by a target protein and the enrichment of the desired genes compared to mono-biotinylated DNA (data not shown). Second, a synthetic streptavidin gene with a reduced GC content (from 68 to 54%) was designed and used to improve the efficiency and specificity of the PCR amplification step. Other genetically engineered streptavidin variants (19,20) may also be advantageous in our system.

In vitro selection of a DNA-displayed random peptide library

To test whether the improved DNA display system was effective in screening peptide ligands, the anti-FLAG M2 antibody was used as bait for selection. The FLAG peptide is a widely used fusion tag specifically designed to facilitate rapid purification of proteins by immunoaffinity chromatography (21). Its usefulness has been shown in many fields, such as a large-scale protein–protein interaction analysis of the yeast proteome (22). The M2 antibody has been used as bait for a phage-displayed library containing ~10^6 random decapeptides (23).

We endeavored to screen diverse peptides that bind to the M2 antibody from a DNA-displayed random decapeptide library with greater diversity. The size of a DNA-displayed library depends on the number of compartments, because the concentration of DNA templates is adjusted so that each micelle contains only one DNA molecule on average.

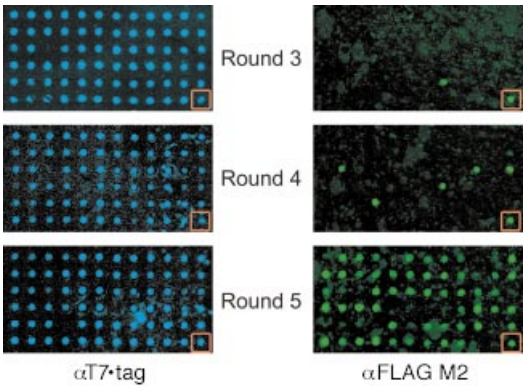

Figure 4. Protein microarray analysis of randomly chosen peptides from rounds 3–5. Attachment of streptavidin-fused peptides on biotin-coated glass slides was confirmed with anti-T7·tag antibody (blue) and the peptides were screened with anti-FLAG M2 antibody (green). Streptavidin-fused FLAG peptide served as a positive control (red squares).

Name	Round	No.	Sequence	% pull-down	K_D (M)
FLAG			DYKDDDDK	59 ± 4	4.3 x 10^{-6}
M2-1	5	15	DNDYKSADAS	74 ± 4	3.7 x 10^{-6}
M2-2		11	SDYKNNDPPL	70 ± 3	7.9 x 10^{-6}
M2-3		7	PHADYKVGDH	69 ± 12	
M2-4		6	DYKNKDDRFP	58 ± 1	
M2-5		6	EYKDRDTHML	49 ± 9	19 x 10^{-6}
M2-6		5	ALPNYKDQDT	54 ± 6	
M2-7		3	HTDYKLLDPP	55 ± 6	
M2-8		2	DHKDHDTPLR	22 ± 3	
M2-9		2	DPLDYKFMDS	32 ± 1	
M2-10		2	LLDYKMIDVP	53 ± 5	
M2-11		2	YQDYKLADTS	29 ± 5	
M2-12		1	HNHDYKMEDT	34 ± 4	
M2-13		1	QLPDYKNYAP	22 ± 6	
M2-14		1	HYDYKLLDSF	37 ± 6	
M2-15		1	TNDYKMRDP	40 ± 8	
M2-16		1	DYKSTDPYWP	33 ± 0.3	6.6 x 10^{-6}
M2-17		1	DYKLRAPVDP	15 ± 0.9	
M2-18		1	QYKMNDTWQD	23 ± 2	
M2-19		1	YKFFDSNRID	7.4 ± 0.1	
M2-20	4	1	IDADYKDYDP		
M2-21		1	DNRDYKMEDT		

Figure 5. Amino acid sequences of selected clones. Critical residues for binding to M2 are shown in red and the number of clones containing each sequence is indicated. Recovery of DNA–peptide conjugates by pull-down assay was determined using quantitative real-time PCR (mean ± SD). Dissociation constants (K_d) were measured by SPR spectroscopy. Five green spots in round 4 in Figure 4 were identified as M2-1, M2-3, M2-6, M2-20 and M2-21. A single spot at round 3 was M2-2.

Addition of the wheat germ *in vitro* transcription/translation mixture to stirred mineral oil produced a water-in-oil emulsion. The droplet diameter ranged from 2 to 6 μm with a sharp peak at ~4 μm. Accordingly, 1 ml of emulsion provided 10^9–10^{10} compartments, each of which was expected to contain a single gene.

The DNA-displayed random decapeptide library was captured on anti-FLAG M2 antibody–agarose beads, which were washed and eluted with FLAG peptide. The simplicity of the selection procedure allowed two rounds of affinity selection per day. After five rounds, DNAs were amplified, translated *en masse* and analyzed by dot blot analysis using the M2 antibody. The fraction of peptides capable of binding to the M2 antibody increased in each round and reached saturation by round 5 (Fig. 3). Protein microarray analysis of randomly chosen clones from rounds 3–5 showed that most peptides from round 5 bound to the M2 antibody (Fig. 4). These results and previous reports indicate that protein microarrays can be used for secondary screening of peptide candidates obtained by affinity selection (17,24).

Cloning and characterization of the selected peptides

Sequence analysis of randomly chosen clones from round 5 revealed that 69 out of 71 clones could be grouped into 19 distinct 'FLAG-like' sequences (Fig. 5). Residues D, Y, K and D in the first, second, third and sixth positions of the original FLAG sequence (DYKDDDDK) (23) were conserved at frequencies of 81, 97, 100 and 97%, respectively. Other non-essential residues at the fourth, fifth, seventh and eighth positions were conserved at 17, 0, 8 and 0%, respectively. Only two clones from round 5, encoding AGNCMRHDTH and PSHLCNPRPL, were false positives. They were neither included in the FLAG-like consensus group nor recovered in the pull-down assay (data not shown). Thus, DNA display selected a more diverse set of peptides (21 sequences) (Fig. 5) than phage display (4 sequences) (23). The percentage of false positives was only 3%, indicating that undesired peptides were almost completely excluded.

Binding affinities of selected peptides were investigated by pull-down assays and surface plasmon resonance (SPR) spectroscopy. In the pull-down assays, each DNA–peptide conjugate was produced independently and captured on anti-FLAG M2 antibody–agarose beads. Then the beads were washed and eluted with FLAG peptide. Recovery rates (Fig. 5, % pull-down) roughly correlated with the frequency of clones, and those of three frequent peptides (M2-1, M2-2 and M2-3) were significantly higher than that of the original FLAG peptide.

To investigate the binding affinities in more detail, we measured the dissociation constants (K_d) of four synthetic peptides by SPR spectroscopy (Fig. 5). In contrast to the pull-down assay, there was no correlation between K_d and the frequency of clones. Instead, the integrity of critical residues correlated with K_d. The K_d of M2-5 (19 μM), lacking a critical residue for binding to M2, was significantly higher than that of the original FLAG (4.3 μM). The K_d values of the other three peptides, which contained all of the critical residues, were similar to that of the original FLAG.

Advantages of DNA display

Use of a DNA genotype offers several advantages over an RNA genotype. (i) The chemical stability of DNA permits screening for peptides under conditions where RNA is easily degraded. These include peptide ligands for receptors that are localized on membranes or whole cells. We are currently screening peptide ligands for G protein-coupled receptors, a superfamily of seven transmembrane proteins that are expressed on cell surfaces. (ii) The stable DNA display would be beneficial in off-rate selection of high affinity binders such as antibodies (25) and enzyme inhibitors over a long period of time. (iii) The method would be useful for directed evolution of novel proteins that work under severe

conditions, because it is easily combined with PCR-based mutagenesis to produce genetic diversity (26–28). (iv) DNA display is widely applicable to the study of protein–protein and small molecule (drug)–protein interactions. Removal of stop codons from full-length cDNA libraries, which is essential for ribosome display and mRNA display systems, is unnecessary.

In summary, completely *in vitro* construction and selection of a DNA-displayed peptide library were accomplished for the first time. DNA display can overcome the limitations of current display technologies and may prove useful for screening a diversity of peptide ligands and proteins.

ACKNOWLEDGEMENTS

We thank H. Takashima for critical comments on the manuscript and N. Matsumura for experimental advice. This work was supported in part by a Special Coordination Fund from the Ministry of Education, Science and Technology, Japan.

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