DNA Display Selection of Peptide Ligands for a Full-Length Human G Protein-Coupled Receptor on CHO-K1 Cells

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Abstract

The G protein-coupled receptors (GPCRs), which form the largest group of transmembrane proteins involved in signal transduction, are major targets of currently available drugs. Thus, the search for cognate and surrogate peptide ligands for GPCRs is of both basic and therapeutic interest. Here we describe the application of an in vitro DNA display technology to screening libraries of peptide ligands for full-length GPCRs expressed on whole cells. We used human angiotensin II (Ang II)-type 1 receptor (hAT1R) as a model GPCR. Under improved selection conditions using hAT1R-expressing Chinese hamster ovary (CHO)-K1 cells as bait, we confirmed that Ang II gene could be enriched more than 10,000-fold after four rounds of selection. Further, we successfully selected diverse Ang II-like peptides from randomized peptide libraries. The results provide more precise information on the sequence-function relationships of hAT1R ligands than can be obtained by conventional alanine-scanning mutagenesis. Completely in vitro DNA display can overcome the limitations of current display technologies and is expected to prove widely useful for screening diverse libraries of mutant peptide and protein ligands for receptors that can be expressed functionally on the surface of CHO-K1 cells.

Introduction

The superfamily of G protein-coupled receptors (GPCRs) [1] is the largest and most diverse group of cell-surface proteins involved in signal transmission. Although a large number of GPCRs has been identified in the human genome project [2,3], more than 100 of them have no known physiologically relevant ligand yet [4,5], and they are classified as orphan GPCRs. Since GPCRs are major targets for today’s drugs [6], the search for cognate and surrogate peptide ligands for GPCRs is of both basic and therapeutic interest [7,8]. Conventional analysis of the specificity of the interaction between GPCRs and peptide ligands involves the mutation of individual amino acids by peptide synthetic methods (e.g., Ala-scanning), followed by measurement of binding affinity or receptor activation. However, the sequence space that can be searched with this standard strategy is quite limited.

As a powerful alternative strategy, phage display has been used to screen peptides that bind to GPCRs expressed on mammalian cells [9–13], but the library sizes and the sequence varieties in a phage library are limited by the transformation efficiency and biological constraints of the host bacteria. This limitation can potentially be overcome by using totally in vitro selection systems, such as ribosome display and mRNA display, which employ cell-free protein synthesis [14–17]. Recently, mRNA display was used to screen peptide ligands that bind to the N-terminal extracellular domain of a class B GPCR immobilized on beads [18], but such an RNA-tagging method requires strictly RNase-free conditions and cannot easily be applied to selection targeting full-length GPCRs expressed on the cell surface.

We have previously developed a DNA display system called STABLE (STreptAvidin-Biotin Linkage in Emulsions) [19–21], in which streptavidin-fused peptides are linked with their encoding DNA via biotin labels in a cell-free transcription/translation system compartmentalized in water-in-oil emulsions. This method allows completely in vitro selection of a stable DNA-tagged peptide library with large diversity in the presence of RNase. In this study, we applied the DNA display system to in vitro selection of peptide ligands for a full-length GPCR expressed on whole cells. As a model to test our screening strategy, we used a well-known GPCR, human angiotensin II (Ang II)-type 1 receptor (hAT1R), which is significantly involved in cardiovascular diseases. Under improved selection conditions using hAT1R-expressing mammalian cells as bait, the Ang II gene was enriched from model libraries (1:100 or 1:10,000 mixture of streptavidin-fused Ang II and streptavidin genes). Further, various Ang II-like peptides were successfully selected from randomized peptide libraries, and their binding...
activity and biological function were characterized to elucidate the sequence-function relationship of hAT1R ligands.

**Results**

**Strategy for in vitro selection of GPCR-ligands**

We improved and applied the STABLE DNA display system [19–22] for in vitro selection of GPCR-ligands on whole cells (Fig. 1). In this system, the linkage of DNA (genotype) and peptide (phenotype) was accomplished in water-in-oil emulsions containing an in vitro transcription/translation system, in which one DNA molecule was caged in each reversed micelle on average [23]. A stable binding of streptavidin with biotin was used as the connector between DNA and its translated products [19]. The number of DNA-peptide conjugates in a library (i.e., library size) is comparable with the number of emulsion droplets ($10^8$–$10^{10}$ per 1 ml of emulsion). The DNA-displayed peptide library was incubated with GPCR-expressing cells in the presence of a GRGDS pentapeptide to inhibit undesired binding of an RGD-like sequence within streptavidin to integrins on the cell surfaces [24]. We also added 0.5 M sucrose to the binding buffer to inhibit internalization of agonist peptides by receptor-mediated endocytosis [25]. Furthermore, in order to repress the large background of cell-surface proteins, glycans and lipids, the library was pre-incubated with ‘Mock’ cells without recombinant GPCR to remove nonspecific binders before incubation with the GPCR-expressing cells (not shown in Fig. 1). A Chinese hamster ovary cell line CHO-K1 is suited for this purpose, because CHO-K1 is a preferred non-human cell line for the efficient and stable expression of a variety of recombinant human proteins including GPCR [26]. Finally, we used a photocleavable 2-nitrobenzyl linker [27] between DNA and peptide for rapid and efficient recovery of selected DNA from DNA-peptide conjugates bound to GPCRs by means of simple photocleavage [22].

**Enrichment of angiotensin II genes on the hAT1R-expressing cells**

As a model GPCR and its ligand, the human angiotensin II type 1 receptor (hAT1R) and octapeptide angiotensin II (Ang II; Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸) were used here. Stable expression of recombinant hAT1R with a C-terminal c-myc tag on CHO-K1 cells was confirmed by immunostaining with anti-c-myc antibody (Fig. 2A). The binding of Ang II to the hAT1R/CHO-K1 cells was confirmed by radio ligand binding assay: the dissociation constant and the number of ligand binding sites were estimated as $K_d = 8.2$ nM and $8.3 \times 10^5$ binding sites/cell, respectively (data not shown). In addition, the function of the recombinant hAT1R was confirmed by monitoring the change in intracellular calcium in response to Ang II (Fig. 2B). These results indicate that a sufficient amount of the model GPCR, hAT1R, was expressed on cell membranes in active form(s), and thus, hAT1R can serve as a bait receptor protein for further in vitro selection experiments.

To confirm whether not only Ang II peptides, but also streptavidin-fused Ang II proteins can bind to hAT1R, streptavidin-Ang II genes with the generally-used linkers were in vitro transcribed and translated, and the products were incubated with the hAT1R/CHO-K1 cells. It was found that the peptide fused with streptavidin through a helical linker [28] (four repeats of ‘GAAAK’) efficiently and specifically bound to the hAT1R/CHO-K1 cells, while the fusion protein with a Gly-rich flexible linker (five repeats of ‘SGGGG’) nonspecifically bound to both the hAT1R/CHO-K1 and the Mock CHO-K1 cells, and the fusion


**Figure 2.** Stable expression of a model GPCR, the recombinant human angiotensin II type 1 receptor (hAT1R) with the C-terminal c-myc tag, on CHO-K1 cells. (A) Immunofluorescence staining of hAT1R-c-myc-expressing CHO-K1 cells (hAT1R/CHO-K1, left) and CHO-K1 cells transfected with the empty vector alone (Mock/CHO-K1, right) with anti-c-myc antibody (green). Nuclei were stained with PI (red). (B) Confirmation of the function of hAT1R-c-myc by monitoring changes in intracellular Ca²⁺ in response to angiotensin II (Ang II). Fura-2/AM-loaded hAT1R/CHO-K1 or Mock/CHO-K1 cells were exposed to 1 nM Ang II at the point indicated by the arrow. The results are expressed as Fura-2 fluorescence ratio (340/380 nm).

**Figure 3.** Construction and enrichment of the streptavidin-fused angiotensin II (STA-Ang II) gene in multiple rounds of DNA display selection on hAT1R/CHO-K1 cells. (A) A schematic representation of the DNA template for *in vitro* transcription/translation. DNA was labeled during PCR with photocleavable biotin [22] at the upstream ends and with fluorescein at the downstream ends, using labeled primers. The translated open reading frame consists of sequences for a T7-tag, streptavidin (STA), a peptide linker, and Ang II gene. The 5′UTR fragment contains T7 promoter. (B) Reaction mixtures containing 1:100 or 1:10,000 molar ratio of STA-Ang II: STA genes were emulsified. The DNA after each round of selection was PCR-amplified with a fluorescein-labeled primer and analyzed by 15% PAGE with an imaging analyzer. doi:10.1371/journal.pone.0030084.g003

**Figure 4.** Characterization of the selected peptides.

**In vitro selection of randomized peptide libraries**

Next, we applied the DNA display method to selection of randomized peptide libraries on whole cells. Since early studies using synthetic analogues of Ang II revealed that Arg², Tyr⁴ and Phe⁸ in Ang II directly interact with hAT1R [29–31], the three residues were fixed in the randomized Ang II library-I. The DNA-displayed randomized peptide library-I was captured on the hAT1R/CHO-K1 cells, which were then washed, and exposed to UV irradiation for elution. After five rounds of selection, DNA was PCR-amplified and cloned. Randomly chosen clones were analyzed by DNA sequencing, and the binding activity of each distinct, in-frame clone was further confirmed (see next section for details). Consequently, five Ang II-like sequences were obtained (Fig. 4A, Binding, +). Not only fixed residues Arg², Tyr⁴ and Phe⁸, but also the residues His⁸ and Pro⁷ of the original Ang II sequence were conserved at frequencies of 100% (Fig. 4B). Other residues Arg⁴ and Val⁸ did not appear (0%) in the selected clones, while Leu⁵ was also highly conserved (Fig. 4B).

To investigate whether the residues Arg², Tyr⁴ and Phe⁸ in Ang II fixed in the library-I are truly essential for binding to hAT1R, we further constructed and screened the randomized Ang II library-II, in which the 2nd, 4th and 8th positions were fixed in the randomized Ang II library-I. Twelve out of 12 peptides specifically bound to hAT1R/CHO-K1 cells. LI5-7 and LI5-8 nonspecifically bound to the hAT1R/CHO-K1 cells with the empty vector alone, as well as the hAT1R/CHO-K1 cells. LI5-5, LI5-9, LI5-9 and LI5-10 (not shown) did not bind to hAT1R/CHO-K1. Although nonspecific binding to the Mock/CHO-K1 cells was also seen for LI5-1 to LI5-4, especially for LI5-2, each band for the Mock/CHO-K1 cells is weaker than that for CHO-K1 cells. LI5-1 to LI5-4 bind to hAT1R/CHO-K1 cells, which were then washed, and residual proteins produced independently and captured on the hAT1R-expressing CHO-K1 cells. The cells were washed, and residual proteins were detected by Western blotting. As shown in Figure 6, 12 out of 18 peptides specifically bound to hAT1R/CHO-K1 cells. LI5-7 and LI5-8 nonspecifically bound to the Mock/CHO-K1 cells with the empty vector alone, as well as the hAT1R/CHO-K1 cells. LI5-5, LI5-9, LI5-9 and LI5-10 (not shown) did not bind to hAT1R/CHO-K1. Although nonspecific binding to the Mock/CHO-K1 cells was also seen for LI5-1 to LI5-4, especially for LI5-2, each band for the Mock/CHO-K1 cells is weaker than that for

**Characterization of the selected peptides**

As mentioned above, we first investigated the binding activities of streptavidin-fused peptides selected from the library-I (Fig. 4A) and the library-II (Fig. 5A). Each streptavidin-fused peptide was produced independently and captured on the hAT1R-expressing CHO-K1 cells. Then the cells were washed, and residual proteins were detected by Western blotting. As shown in Figure 6, 12 out of 18 peptides specifically bound to hAT1R/CHO-K1 cells. LI5-7 and LI5-8 nonspecifically bound to the Mock/CHO-K1 cells with the empty vector alone, as well as the hAT1R/CHO-K1 cells. LI5-5, LI5-9, LI5-9 and LI5-10 (not shown) did not bind to hAT1R/CHO-K1. Although nonspecific binding to the Mock/CHO-K1 cells was also seen for LI5-1 to LI5-4, especially for LI5-2, each band for the Mock/CHO-K1 cells is weaker than that for
hAT1R/CHO-K1 cells, respectively, and thus they were served as candidates for further characterization.

To clarify whether the selected 12 peptides bind to hAT1R in the same manner as Ang II, we next performed competitive binding assays. Except for LI5-3, the peptides bound to hAT1R/CHO-K1 cells only in the absence of the Ang II competitor (Fig. 7), indicating that the selected peptides do interact with hAT1R at the Ang II-binding site. Again, some content of LI5-2 non-specifically bound to the cell-surface (Fig. 6) and thereby seems not to be completely competed out by Ang II (Fig. 7). A reason may be a relatively higher hydrophobicity of LI5-2 peptide with Trp1 in comparison with other peptides having hydrophilic amino acids such as Asn, His or Arg at the position 1.

Furthermore, IC50 values were determined by means of competitive binding assays at various concentrations of synthetic peptides (see MATERIALS AND METHODS and Figure S1). (B) Sequence logos representation [45,46] of the peptide sequences with specific binding activity (+). The height of each column reflects the bias from random of particular residues. Fixed residues are shown in gray. Polar amino acids containing an amide group (Q, N) and the rest of them are shown in purple and green, respectively, basic charged residues in deep blue, and hydrophobic residues in black.

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Discussion

In this study, completely in vitro selection of peptide ligands for a full-length GPCR expressed on whole cells was accomplished by using an in vitro DNA display technology. With Ang II receptor as a model GPCR, wild-type and mutated Ang II peptides were successfully selected from model libraries and randomized peptide libraries, respectively. As shown in Fig. 3, when only Ang II was the binding sequence in the doped library, the Ang II sequence was selected from the model library. However, no wild-type sequence was selected from the randomized libraries, though a variety of Ang II-like peptides were identified (Figures 4 and 5).

The constrained random libraries contain a variety of binding sequences, some of which become fixed by random genetic drift
However, LII3-5 with Lys2 retains affinity as high as that of substitution at positions 2, 4, 6 and 7, respectively ([binding affinity of synthetic Ang II to hAT1R was affected by Ala hAT1R. This result is consistent with the previous finding that the affinity of [Lys2]Ang II for rat AT1R was lower than that of wild-type Ang II.]

The analysis of the selected peptides revealed that the residues Arg2, Tyr4, His6 and Pro7 in Ang II are important for binding to hAT1R. This result is consistent with the previous finding that the binding affinity of synthetic Ang II to hAT1R was affected by Ala substitution at positions 2, 4, 6 and 7, respectively ($K_d > 10 \mu M$) [32]. However, LII3-5 with Lys2 retains affinity as high as that of wild-type Ang II (Fig. 5A), which is inconsistent with the previous study [33]; the affinity of [Lys2]Ang II for rat AT1R was lower than that of wild-type Ang II.

While the residue Phe8 is important for high agonist activity [29,31], a series of Ang II analogs mutated at the C-terminal position 8 (e.g., Ile8 and Leu8) exhibit antagonist activity [34,35]. However, the selected peptides LII3-2 with Ile8 and LII3-4 with Leu8 possess agonist activity (Fig. 8C,D), indicating that nonconserved positions 1 and 3 are also important in determining the function of Ang II-like peptides. Furthermore, the shapes of the calcium fluxes for the mutated peptides are somewhat different from that for wild-type Ang II. It may reflect a possible different signaling by the selected peptides, because there is a growing body of literature suggesting that different ligands bind to and activate the same GPCR through different signaling pathways [36,37]. Thus, further studies for selection of peptide ligands have the potential to contribute to the GPCR biology in the future.

The use of DNA display for searching peptide ligands offers several advantages over other display technologies, such as phage display and mRNA display. First, as mentioned in the introduction, the size of DNA display libraries based on cell-free protein synthesis is usually greater than that of phage libraries using bacterial expression, and the chemical stability of DNA permits selection targeting full-length GPCRs expressed on cell-surface, where RNA would be easily degraded. Further, each peptide in the conventional phage display and mRNA display libraries should be linked with its genotype at the C-terminus, while a peptide can be fused with both N- and C-terminals of streptavidin in DNA display [38]. The free N- or C-terminals of peptide ligands is often important for their biological activities. Finally, the use of the reconstituted transcription/translation system in DNA display allows various applications; for example, the incorporation of unnatural amino acids [39,40] for in vitro selection of peptide mimetic compounds. Thus, the method we describe should prove useful not only for the mutation analysis of ligand/receptor interaction, but also for screening of agonists and antagonists for disease-related GPCRs that can be expressed functionally on the surface of CHO-K1 cells, and for the identification of peptide and protein ligands for orphan GPCRs except for those with non-peptide endogenous ligands.

**Materials and Methods**

**Preparation of GPCR-expressing mammalian cells**

The hAT1R gene (1080 bp) [41] was amplified from a human liver cDNA library (from Prof. J-I. Inoue; Institute of Medical Science, University of Tokyo) by means of PCR with *Ex Taq* DNA polymerase (TaKaRa Shuzo) using the primers hAT1R-F and hAT1R-R [all primer sequences used in this study are listed in Table S1]. The PCR product was digested with EcoRI and XbaI and then cloned into the identical restriction-enzyme sites of a vector pEF1/myc-HisA (Invitrogen) comprising an EF1α promoter and fusion-tag sequences encoding C-terminal c-myc and polyhistidine tags. The resulting plasmid pEF1-hAT1R-MycHis was confirmed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems), purified with an endofree plasmid maxi kit (Qiagen), and used for transformation of the mammalian cell line CHO-K1 [a Chinese hamster ovary cell line purchased from RIKEN Cell Bank (Ibaraki, Japan) in 2000] with lipofectamine 2000 (Invitrogen). As a control, CHO-K1 cells were also transfected with the empty vector alone (Mock/CHO-K1). The transfected cell lines were kept under selection pressure of 400 µg/ml G418. The expression of the recombinant hAT1R was confirmed by Western blot analysis of cell lysates using mouse anti-c-myc monoclonal antibody (MBL) and an ECL plus Western blotting analysis system (Amersham Biosciences).
Immunofluorescence staining

The hAT1R-expressing mammalian cells were plated on a micro cover glass sunk in complete medium [10% (v/v) FBS, 90% (v/v) Ham’s F-12, 100 units/ml penicillin, 100 μg/ml streptomycin, 400 μg/ml G418] and incubated for 48 h at 37°C in an atmosphere of 5% CO2 in air. Prior to immunostaining, cells were fixed in PBST with 3.7% paraformaldehyde for 10 min at room temperature, treated with 0.1 mg/ml RNase A (DNase-free; Invitrogen) for 20 min at 37°C, and blocked in PBS with 1% BSA for 30 min at room temperature. Antibodies were diluted in PBS with 1% BSA. Immunofluorescence staining was performed with mouse anti-c-myc monoclonal antibody (Santa Cruz Biotechnology) for 45 min at room temperature. The cover glass was washed three times with PBS, incubated with goat anti-mouse Alexa Fluor 488-conjugated antibody (Molecular Probes) for 50 min and washed three times with PBS. For nuclear staining, the cover glass was incubated with 500 nM propidium iodide (PI; Molecular Probes) in 2×SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) for 5 min at room temperature and washed three times with 2×SSC. Then the cover glass was inverted and set on a slide glass. The slides were viewed with a confocal microscope (Nikon Eclipse E600 microscope and Bio-Rad Radiance 2000 scanning system) for Alexa Fluor 488 (495/519 nm) and PI (536/617 nm).

Radio ligand binding assays

Cells were harvested in 48-well plates (IWAKI) at a density of approximately 6×10⁵ cells/well. The cells were blocked with Ham’s F12 medium containing 1% BSA, then [³²P]Sar¹Ile⁶-Ang II was added (2,000 Ci/mmol; Amersham Biosciences; final concentration in 150 μl final volume ranging from 0.05 nM to 20 nM). After incubation for 30 min at room temperature, the cells were washed three times with 500 μl of PBS containing 1% BSA and radioactivity was counted with a MINAXI auto-gamma 5000 scintillation counter (Canberra-Packard). Values for receptor capacity and affinity were obtained by Scatchard analysis.

Calcium imaging

The intracellular concentration of calcium [Ca²⁺]i was measured by incubating hAT1R-expressing cells with the fluorescent Ca²⁺ indicator, 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranoyl]2-(2-amino-5-methylphenoxy)ethane-N,N,N,N⁹-tetraacetic acid penta-acetoxymethyl ester (Fura-2/AM) as previously described [42]. Briefly, the cells in fresh BSS (balanced salt solution; 20 mM HEPES, pH 7.3, 130 mM NaCl, 5.4 mM KCl, 5.5 mM d-glucose, 1.8 mM CaCl₂ and 0.8 mM MgSO₄) were loaded at 37°C for 30 min with 5 μM Fura-2/AM, conditioned in flowing BSS at 37°C, then exposed to the stimulating reagent Ang II or synthetic peptides for selected clones by changing the BSS flow to peptide-containing BSS flow for 30 s. The fluorescence of the cells was then measured at the emission wavelength of 510 nm with sequential excitation at 340 nm and 380 nm. The 340/380 ratio can be converted to [Ca²⁺]i according to the formula presented by Grynkiewicz et al. [43].

Preparation of DNA libraries

Two libraries of streptavidin-fused random peptides were amplified by three steps of PCR from a pSta4-derived plasmid [20] carrying a streptavidin gene with the N-terminal T7-tag and the C-terminal helical peptide linker by the 1st step PCR with ExTag DNA polymerase, using forward primer T7tagF-M and reverse primer HLA-SG-RYF-NYN-R (for library-I) or HLA-SG-MTH-NTN-NYN-R (for library-II) (Table S1). Each PCR product was re-amplified using forward primer T7F-M and reverse primer T7R-M (library-I) or T7R-M2 (library-II), and finally re-amplified using photocleavable biotin (PCB)-labeled T7F and T7R primers. The PCR products were purified with a...
Preparation of DNA-displayed peptide libraries

In vitro transcription/translation reactions in water-in-oil emulsions were performed as previously described [19,23] with the following modifications. The PURE system classic II kit (Post Genome Institute Co., Ltd.) based on a reconstituted E. coli translation/transcription system [44] was used as the water phase, and mineral oil (Nacalai Tesque) containing 4.5% (v/v) Span85 (Nacalai Tesque) and 0.5% (v/v) Tween80 (Nacalai Tesque) was used as the oil phase. 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. The amounts of emulsion were 10 ml (including 500 μl of PURE system) for the first round, 4 ml (200 μl) for the second round, 2 ml (100 μl) for the third round, and 1 ml (50 μl) for further rounds. Thus, the numbers of DNA molecules in each library were ~6 × 10^10 for the first round, ~6 × 10^9 for the second round, ~3 × 10^8 for the third round, ~2 × 10^7 for the fourth round, and ~3 × 10^6 for the fifth round. The emulsions were incubated for 2 h at 37°C. To recover the water phase, the emulsions (1 ml) were spun at 2,000 g for 10 min. The cell lysates were analyzed by 15% SDS-PAGE and Western blot analysis using anti-T7 tag antibody (Novagen) followed by HRP-conjugated secondary antibody (Chemicon). The streptavidin-fused peptides with T7 tag were quantitatively detected by an ECL chemiluminescence kit and Hyperfilm ECL (GE Healthcare) [47].

Streptavidin-fused peptide binding assays

Streptavidin-fused peptides for selected clones were prepared separately using the PURE system classic II kit with 20 nM DNA for 1 h at 37°C. The streptavidin-fused peptides were incubated with the hAT1R-expressing cells for 10 min at room temperature in the presence 0–200 nM synthetic peptides (see next section) as competitors, and washed as described in the previous section. Then the binding proteins were recovered using 0.5 ml of lysis buffer (1% protease inhibitor cocktail and 4 M urea) on ice for 30 min. The cell lysates were analyzed by 15% SDS-PAGE and Western blot analysis using anti-T7 tag antibody (Novagen) followed by HRP-conjugated secondary antibody (Chemicon). The streptavidin-fused peptides with T7 tag were quantitatively detected by an ECL chemiluminescence kit and Hyperfilm ECL (GE Healthcare) [47].

Supporting Information

Figure S1 Competition curves for selected peptides. The procedures of the LI5-series were synthesized as follows: variations in the presence of various concentrations of synthetic peptides LI5-1 (filled circles), LI5-2 (filled squares), LI5-3 (open circles), LI5-4 (open squares) and LI5-5 (open triangles), washed and analyzed by 15% SDS-PAGE and Western blot analysis. For details, see MATERIALS AND METHODS.

Table S1 Oligonucleotide sequences. N = A, C, G or T; D = A, G or T; M = A or C; R = A or G; K = G or T. T7F labeled with photocleavable biotin (PCB) and T7R labeled with fluorescein were used for preparation of labeled DNAs.

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Author Contributions
Conceived and designed the experiments: ND TN NM HY. Performed the experiments: NY HM YY TN. Analyzed the data: ND NY HM YY TN. Wrote the paper: ND KH HY.

References


