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Screening of α-helical peptide ligands controlling a calcineurin-phosphatase activity

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Abstract—In this paper, we describe an application of 202-membered fluorescently labeled peptide library designed to take an α-helix secondary structure. As a proof-of-concept experiment, a calmodulin (CaM)/calcineurin (Cn) pair was chosen to screen α-helical peptide ligands that tightly bind to CaM and also control enzymatic functions of Cn. Three peptides were successfully selected from the library by assaying Cn-phosphatase activities and peptide–CaM interactions (dual check process). The strategy using a designed peptide library shows real promise as a peptide-based high-throughput screening system.

The elucidation of genome sequences of various organisms has been successfully completed. In the post-genome-sequencing era, high-throughput protein-detecting techniques are widely developed for proteomic analyses in biotechnological and biomedical researches.[1–5] In such technologies, a large number of capture agents that selectively bind to proteins of interest are arrayed in a variety of dry, wet, and semi-wet formats. We are now focusing on the use of designed peptides for high-throughput analyses of protein interactions and properties.[6–12] Peptides with suitable secondary and/or tertiary structures are promising candidates as protein capturing agents, because (i) structured peptides are easier to design and synthesize than antibodies or recombinant proteins, (ii) they can mimic protein–protein interactions, and (iii) data analyses based on peptide arrays can elucidate the binding properties of proteins to peptides. Thus, the designed peptide arrays have great promise for the realization of a high-throughput protein microarray system for proteomics and ligand screening applications.

In this paper, to demonstrate an application of the designed peptide library, we attempted a model of ligand screening assay in a microtiter plate format prior to using them in a chip format. Unlike in normal ligand screenings, we attempted to find peptide ligands that not only bind to a target protein but also control the target protein's function. By conducting such an approach, we hoped to identify ligands that tightly bind to a specific binding site of a target protein. Even though it was performed in the microplate format, identifying such ligands would be useful for applications of designed peptide libraries toward the peptide-based chip technology.

The calmodulin (CaM)-calcineurin (Cn) system was initially selected as an activator-enzyme model (Fig. 1A). Cn is a Ca^{2+}–CaM-dependent protein phosphatase. It was assumed that peptides which bind specifically to CaM and compete with CaM binding to activate Cn would be screened by monitoring both Cn phosphatase activities and peptide–CaM interactions. For the purpose of this study, fluorescently labeled peptide library was thus newly designed and synthesized based on the L8K6 sequence, an α-helical peptide known to bind to CaM.[7,12] In the screening procedure, the phosphatase activity of Cn was assayed in a solution containing CaM and the library peptides in order to efficiently screen for ligands that bind to a unique site of CaM. After the screening procedure, the binding constants K_a of the selected peptides were determined by their change in fluorescence. Throughout the assaying procedure, the ligand screening method based on an enzyme activity in conjunction with the designed peptide library having a fluorophore has the following advantages: (i) the peptide library can be easily designed with various

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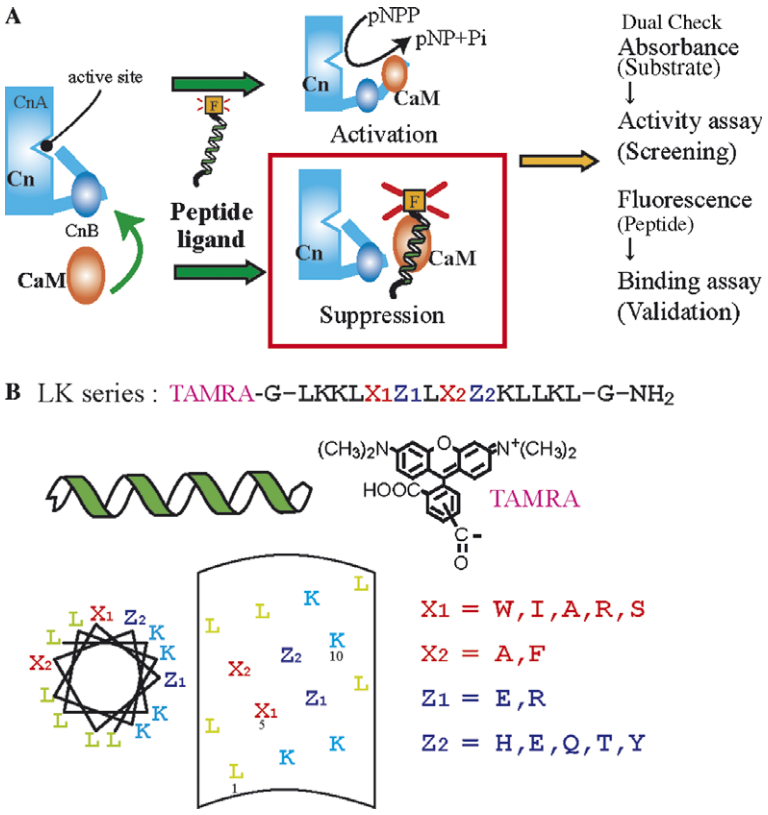

Figure 1. (A) Illustration of the screening of peptide ligands for calmodulin (CaM) which control calcineurin (Cn) phosphatase activity. (B) Strategy for the construction of the library consisting of 202 α-helical peptides. A half part of the library (LK series) is shown. The rest part (LE series) is shown in Figure S1.

charges and/or hydrophobicities systematically within the peptide secondary structures, (ii) binding properties of a target protein can be better characterized by monitoring changes in fluorescence intensity, (iii) ligands that bind to a specific position of the target protein can be more easily selected using a panel of target proteins, and (iv) selected ligands would be promising control tools for study of a signaling pathway and/or a cascade. Thus, this screening strategy using designed peptide library could play an important role not only in the discovery of active structured ligands but also in establishment of a peptide-based high-throughput detection system.

A model activator-enzyme was selected in order to establish the screening strategy for regulating a specific enzyme activity. CaM, an activator protein, consists of 148 amino acids with two EF-hands and binds to cationic amphiphilic α-helical peptides in the presence of Ca^{2+}.[13,14] Cn was selected as the model enzyme. Cn is both a Thr/Ser protein phosphatase and a Ca^{2+}–CaM-dependent enzyme that has an important role in the control of intracellular Ca^{2+} signaling. Because Cn can also dephosphorylate small phosphorylated substrates such as *p*-nitrophenyl phosphate (pNPP) in vitro,[15–17] the Cn phosphatase activity can be measured by increase in absorbance at 405 nm corresponding to *p*-nitrophenol released. The Cn is a heterodimer of one catalytic subunit (CnA, ca. 60 kDa) and one regulatory subunit (CnB, ca. 20 kDa). These two subunits of calcineurin are tightly bound to each other. CnA alone has very low activity and the presence of the B subunit is essential for the highly specific activity of the phosphatase, which is further activated by CaM.

A library consisting of 202 α-helical peptides designed with various charges and/or hydrophobicities and a *N,N,N′,N′*-5(6)-carboxytetramethylrhodamine (TAMRA) at the N-terminus of the sequences was constructed for the ligand screening system. The strategy for the construction of this library is shown in Figure 1B and Figure S1, and Table S1. In the case of the peptides in the LK series, a cationic amphiphilic α-helical peptide, L8K6 (No. 000) with the sequence LKKLLKLL KKLLKL[18] known to bind to CaM in the presence of Ca^{2+}, four residues in the central region of the L8K6 were replaced with various amino acids, that is, the X_1 residue was replaced with W, I, A, R, and S, the X_2 residue was replaced with A and F, the Z_1 residue was replaced with E and R, and the Z_2 residue was replaced with H, E, Q, T, and Y. For the peptides in the LE series, on the basis of the L8K2E4 (No. 201) sequence,[7] an acidic amphiphilic α-helical peptide with the sequence LEKLLELLKELLEL, four residues in the central region of the L8K2E4 were also replaced with various amino acids, that is, the X_1 residue was replaced with W, I, A, R, and S, the X_2 residue was replaced with A and F, the Z_1 residue was replaced with K and R, and the Z_2 residue was replaced with H, R, Q, T, and Y. This designed peptide library was constructed using standard

solid-phase Fmoc-chemistry[19] with manual procedures. The synthetic procedures were optimized in order to use the peptides without HPLC purification. The details are described in the Experimental section of Supporting Information. After synthesis, all the peptides were characterized by HPLC (Fig S2) and MALDI-TOFMS (Table S2). Their purities were sufficient for the following demonstration of the ligand screening, even without laborious HPLC purification steps.

To compare the Cn activities with/without CaM by addition of various library peptides, the Cn phosphatase activity was measured (405 nm absorbance of *p*-nitrophenol released from pNNP) using 96-well microplates (Fig. 2A). For measuring Cn activities in the presence of CaM (Fig. 2B), the relative activities were expressed using the ratio, A/A_{ref}, where A is the activity of Cn with CaM upon addition of each peptide and A_{ref} is the activity of Cn with CaM in the absence of the peptides. This assay screened for peptides that showed relative activities <1, represented by the blue or green lines in the color barcode. Various peptides were selected for which the activity was lower than with CaM alone.

In the case of Cn activities in the absence of CaM (Fig. 2C), the relative activities were also expressed using the ratio, A/A_{ref}, where A is now the activity of Cn upon addition of each peptide and A_{ref} is the activity of Cn

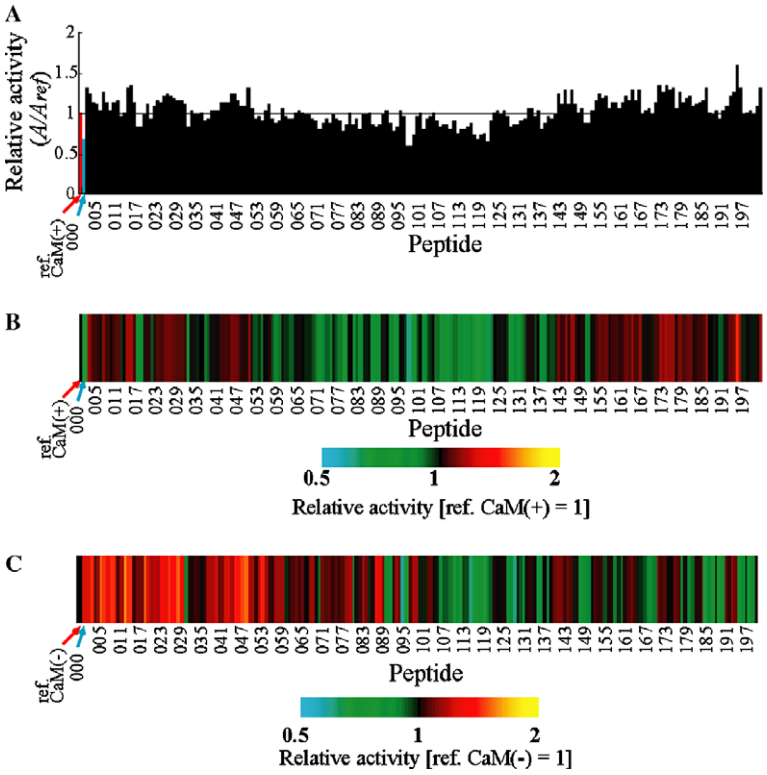

Figure 2. Relative Cn activities with/without CaM by addition of various library peptides using the ratio, A/A_{ref}, where A is the activity of Cn with CaM upon addition of each peptide and A_{ref} is the activity of Cn with CaM in the absence of the peptides ['ref. CaM(+)' = A_{ref}/A_{ref} = 1], and their color barcode. Cn = 0.1 U, CaM = 5 U, [pNPP] = 10 mg/mL, [Peptide] = 1.0 μM in 20 mM Tris–HCl, 1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 150 mM NaCl, and 20 mM PEG2000 (pH 7.4) at 30 °C. (A and B) Relative Cn activities with CaM (graph and color barcode) and (C) without CaM (color barcode).

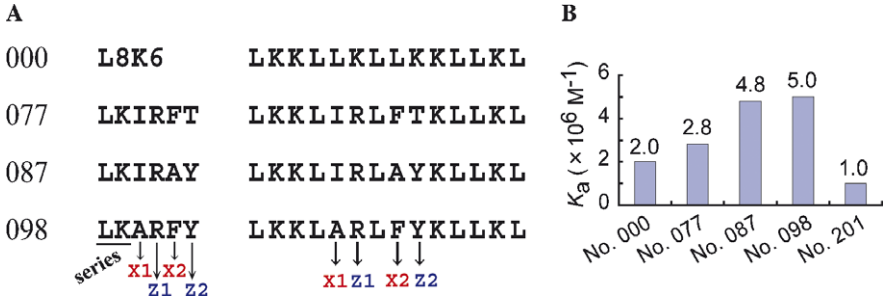

Figure 3. (A) Sequences of the peptides selected by the screening procedures. (B) Binding constants of the representative peptides (Nos. 000, 077, 087, 098, and 201) determined by fluorescent changes of the peptides ([Peptide] = 1.0 μM) upon addition of CaM in 20 mM Tris–HCl, 0.1 mM $CaCl_2$, 150 mM NaCl, and 20 mM PEG2000 (pH 7.4) at 25 °C (2.0 mL scale).

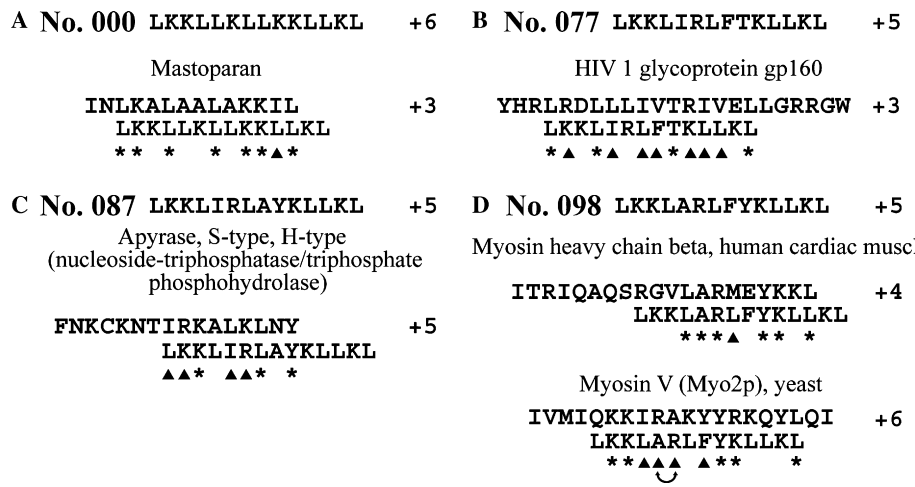

Figure 4. Alignment between the core sequences of the peptides selected from the library and CaM binding sequences in the naturally occurring proteins. The database contained nearly 300 sequences known to bind to CaM, published as of March 2003.[22] The symbol '∗' represents a residue overlap between the sequences analyzed by SIM.[23] The symbol '▲' represents a similar residue overlap between the sequences judged by features of a hydrophobicity, charge, or chemical structures.

alone without the peptides. This assay screened for peptides that showed relative activities ≈1, represented by the black lines in the color barcode, indicating that the selected peptides did not directly bind to the active site of Cn under the conditions lacking CaM. Peptides that met the criteria of both assays (Fig. 2A–C) were selected for further assay, that is, No. 000 as a positive reference, Nos. 077, 087, and 098 as peptides preferable for this ligand screening study (Fig. 3A), and No. 201 as a negative reference showing a lower binding affinity to CaM. Peptides, Nos. 077, 087, and 098 have some common features such as that their Z_1 are Arg residues and their Z_2 are amino acids that contain hydroxyl groups such as Tyr and Thr. We confirmed that all of these peptides except No. 201 have Cn suppression abilities in the larger-scale assay performed in a quartz cell (1 mL scale) (Figure S3). The suppression of Cn activity by Nos. 077, 087, and 098 was almost equal to that of No. 000 (the positive reference) and all of them suppressed Cn activity in a dose-dependent manner. Since Cn alone has a substantial activity, the activity was not suppressed to be zero. The peptides directly bound to CaM for suppressing the Cn activity, because they did not affect the Cn activity in the absence of CaM.

In order to estimate the structural features of the selected peptides, the far-UV CD spectra of the designed peptides were measured, as shown in Figure S4. Representative peptides (Nos. 000, 077, 087, 098, and 201) displayed negative minima with ellipticities at 208 and 222 nm, which are characteristic of an α-helical structure.[20] These results indicated that the peptides themselves were taking an α-helical structure in aqueous solution, although a variety of α-helical propensities were observed depending on the sequences.

To compare the binding constants K_a of the selected peptides with CaM, the fluorescence spectra of TAMRA in these peptides and the subsequent change in fluorescence upon addition of CaM were measured (Fig. 3B). In the case of No. 000 as a positive reference, a ca. 3-fold increase in the fluorescence intensity was observed upon addition of CaM. From the fluorescence change, the binding constant of No. 000 with CaM was calculated as $2.0 \times 10^6\ M^{-1}$ by means of a single site binding equation.[21] The binding constants of Nos. 077, 087, and 098 with CaM were calculated as $2.8 \times 10^6\ M^{-1}$, $4.8 \times 10^6\ M^{-1}$, and $5.0 \times 10^6\ M^{-1}$, respectively. This indicated that these three peptide ligands bind to CaM more tightly than No. 000. In addition, ca. 10-fold increase in fluorescence intensity was observed upon addition of CaM with either Nos. 087 or 098. This suggests that these two peptide ligands may be promising candidates for CaM capture agents in fluorescent assays.

Furthermore, the core sequences of the selected peptides were aligned with those of CaM binding regions in the naturally occurring proteins (Fig. 4). CaM binding sequence data sets were obtained from the calmodulin target database on the Internet.[22] The alignment analysis was performed by SIM on the Internet.[23] SIM is a program that provides a user-defined number of best non-intersecting alignments between two protein sequences or within a sequence. More than seven residues in each peptide core sequence of 14 residues were judged to be similar or the same overlaps to the natural counterparts of CaM binding sequences. This implies that our screening strategy is a promising approach for discovering unique binding sequences.

In conclusion, we demonstrated the successful application of screening for peptide ligands that tightly bind to a target protein and also control the protein's functions. This approach has several advantages such as ease of peptide library design, inclusion of different charges and/or hydrophobicities within the peptides' secondary structures, and robust selection of novel structured ligands for potential use as control tools for a signaling pathway and/or a cascade. In the case of the system studied here, three peptide ligands that not only bound tightly to CaM but also controlled the Cn phosphatase activity were successfully selected for. These peptide li-

gands bound to CaM more tightly than No. 000 (L8K6), the positive reference. We believe that this novel strategy using a designed peptide library shows real promise as a screening system applicable to a microarray system for both drug discovery and diagnostics. Furthermore, combination of the present study and the microarraying technology we developed previously[12] can lead to one of the practical protein-analyzing chip technologies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.09.075.

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