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Effective modification of cell death-inducing intracellular peptides by means of a photo-cleavable peptide array-based screening system

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Intracellular functional peptides that play a significant role inside cells have been receiving a lot of attention as regulators of cellular activity. Previously, we proposed a novel screening system for intracellular functional peptides; it combined a photo-cleavable peptide array system with cell-penetrating peptides (CPPs). Various peptides can be delivered into cells and intracellular functions of the peptides can be assayed by means of our system. The aim of the present study was to demonstrate that the proposed screening system can be used for assessing the intracellular activity of peptides. The cell death-inducing peptide (LNLISKLF) identified in a mitochondria-targeting domain (MTD) of the Noxa protein served as an original peptide sequence for screening of peptides with higher activity via modification of the peptide sequence. We obtained 4 peptides with higher activity, in which we substituted serine (S) at the fifth position with phenylalanine (F), valine (V), tryptophan (W), or tyrosine (Y). During analysis of the mechanism of action, the modified peptides induced an increase in intracellular calcium concentration, which was caused by the treatment with the original peptide. Higher capacity for cell death induction by the modified peptides may be caused by increased hydrophobicity or an increased number of aromatic residues. Thus, the present work suggests that the intracellular activity of peptides can be assessed using the proposed screening system. It could be used for identifying intracellular functional peptides with higher activity through comprehensive screening.

[**Key words:** Peptide array; Screening system; Cell penetrating peptides; Peptide modification; Peptide drug]

Peptides are essential molecules that have various functions in the body. Some peptides play roles inside cells and are called intracellular functional peptides. They have been used as cell death/differentiation inducing factors (1–6) and for theranostics (7): a peptide inhibitor of mutant p53 aggregation, which was designed on the basis of the p53 aggregation site, acts as a rescuer of the activity of p53 (1), a polypeptide derived from G_1/S-specific cyclin D2 protein regulation of the cell cycle (2), among other examples (3–6). Furthermore, intracellular functional peptides have a potential as drug leads. For instance, a synthetic small molecule that mimics the structure of the intracellular peptide that inhibits p53 degradation showed high activity of induction of cancer cell death, and this molecule reached a clinical trial (8). Thus, discovery of effective intracellular functional peptides is expected to contribute to novel drug development.

Gene *Noxa* encodes a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins (9) and plays an important role in apoptosis induced by p53-dependent genotoxic stimuli (9–11). Noxa has 2 functional domains, the BH3 domain and mitochondria-targeting domain (MTD), and each domain has a cell death-inducing activity. As for MTD, it was reported that MTD kills various cancer cells (12) and identified sequences of the cell death-inducing intracellular peptides derived from MTD (KLLNLISKLF, LLNLISKLF, and LNLISKLF) (12,13). These peptides combined with cell-penetrating peptides (CPPs) may be internalized into cancer cells and can kill the treated cells by opening the mitochondrial permeability transition (mPT) pore resulting in a release of mitochondrial calcium to the cytosol (12).

A peptide array is a useful tool for analyzing protein–protein interactions, like those involving antibodies (14), receptors (15), and cytokines (16,17). We have used a cellulose membrane-based peptide array to identify various functional peptides, such as a cell death-inducing peptide (18), a bile acid-binding peptide (19), and cell adhesive peptides (20). We also constructed a photo-cleavable peptide array for assessing solubilized peptides and identified a peptide inhibitor of α-amylase activity (21). Previously, we applied the photo-cleavable peptide array technology (21) and proposed a novel screening system for intracellular functional peptides (22). In this system with the 96-well format, a candidate peptide–CPP complex is synthesized on the cellulose membrane via a photo-cleavable linker in each spot. After UV irradiation, each spot on the array is punched out, and placed in one well of a 96-well plate. After the punched disk is applied to cultured cells, various intracellular functions can be assayed. In the previous paper about this system, we proposed that the cellular uptake of

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the candidate peptide–CPP complex could be predicted by the scatter diagrams of hydrophobicity and the isoelectric point of candidate peptides (22). Nonetheless, this system has not been used for screening of intracellular functional peptides with higher activity so far.

In the present study, we demonstrated that peptide function can be assessed using the proposed screening system for assessing the intracellular activity of peptides. The cell death-inducing peptide (LNLISKLF) identified in the MTD of the Noxa protein (13) served as an original peptide sequence.

MATERIALS AND METHODS

Synthesis of photo-cleavable peptide arrays This synthesis was reported previously (21). A cellulose membrane (grade 542; Whatman, Maidstone, UK) was activated using β-alanine as the N-terminal basal spacer. An Fmoc-Photo-Linker (sc-294977A; Santa Cruz Biotechnology, TX, USA) served as a photo-cleavable linker for the Fmoc peptide synthesis. The linker conjugated candidate peptides with cellulose. An Fmoc-activated amino acid (0.25 mol/L) was spotted on the membrane by means of a peptide auto-spotter (ASP222; Intavis, Cologne, Germany), following the manufacturer's instruction with some modifications. After addition of the first residue, the remaining amino groups were blocked twice with 5% acetic anhydride for 15 min. At each elongation step, the membrane was deprotected using 20% piperidine and then washed thoroughly with N,N′-dimethylformamide, followed by a wash with methanol. After final deprotection, the side chain-protecting groups were removed for 2.5 h by means of a mixture of trifluoroacetic acid (TFA, A00025; Watanabe, Hiroshima, Japan), m-cresol (034-04646; Wako, Osaka, Japan), 1,2-ethanedithiol (A00057; Watanabe), thioanisole (T0191; Tokyo Chemical Industry, Tokyo, Japan) at a ratio of 40:1:3:6. Finally, the membrane was washed thoroughly with diethyl ether and methanol, consecutively. Three spots for each peptide sequence were deposited on each membrane. By analyzing each residue of peptides with bromophenol blue (BPB), we confirmed quality of the peptides synthesized by means of the peptide auto-spotter.

The release of peptides from the photo-cleavable peptide arrays Each peptide on a photo-cleavable peptide array was cleaved from the solid phase by irradiation with UV at 365 nm. The peptide arrays were dried completely at room temperature, and irradiated with UV at 365 nm for 3 h using a transilluminator (DT-20LCP; Atto, Tokyo, Japan) (21). After that, each spot on the array was punched using a biopsy punch (diameter, 6 mm; KAI Corp., Tokyo, Japan). Each resulting peptide-containing disk (peptide spot) was placed in a single well of a 96-well plate with a filter (MSRLN0410; Merck Millipore, Darmstadt, Germany) and the peptide was released into 150 μL of serum-free Dulbecco's modified Eagle medium (DMEM, 08458-16; Nacalai Tesque, Kyoto, Japan) at 37°C for 1 h. After release of each peptide, the medium containing the peptide was filtered into a 96-well plate by vacuum filtration (MultiScreen HTS Vacuum Manifold; Merck Millipore) to remove various insoluble materials, and was subsequently used for the assay (22).

Cell culture MCF-7 cells were maintained in 75 cm^2 flasks (658170; Greiner Bio-One, Frickenhausen, Germany) cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Biosera; Nuaille, France), and 1% Penicillin/Streptomycin (PS, 168-23191; Wako). The cells were cultured in a humidified 5% CO_2 incubator at 37°C to approximately 80% confluence.

The cytotoxicity assay MCF-7 cells (10^4 cells/well) were plated in wells of 96-well plates (TR5003; Nippon Genetics Co., Ltd., Tokyo, Japan) and cultured for 24 h. The culture medium was removed, and we added the medium containing peptides. The plate was incubated for 3 h in the CO_2 incubator. After that, the cell viability was determined with the Cell Counting Kit-8 (347-07621; Dojindo, Kumamoto, Japan). The peptides (LNLISKLF-R8, LNLIFKLF-R8, LNLIVKLF-R8, LNLIWKLF-R8, and LNLIYKLF-R8) were purchased from Cs Bio (Shanghai) Ltd. These peptides were over 95% purity.

Measurement of intracellular calcium For Ca^{2+} measurements in the cytosol, MCF-7 cells (5.0×10^4 cells/dish) were cultured in a glass-based dish (3910-035; Iwaki, Tokyo, Japan) and loaded with Fluo-4-direct (F10471; Thermo Fisher Scientific, MA, USA) at a concentration following the manufacturer's instructions for 30 min, followed by washing with fresh Ca^{2+}-free Krebs-ringer modified buffer [KRB: 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L Na_3PO_4, 1 mmol/L $MgSO_4$, 5.5 mmol/L glucose, and 20 mmol/L HEPES (pH 7.4), at 37°C] and the addition of KRB-containing peptides (12). Time-lapse images were obtained at 488-nm excitation using Spinning Disk Confocal system (X-Light V1; Crest Optics, Rome, Italy) with microscope (IX81; Olympus, Tokyo, Japan) at 10-s intervals for 5 min to visualize Fluo-4-direct. The fluorescein intensity was measured by ImageJ software.

Statistical analysis Data are presented as mean values and standard deviation (SD), and Student's t test was used for evaluating statistical significance for comparison. A value less than 0.05 ($p < 0.05$) indicated statistical significance.

RESULTS AND DISCUSSION

Validation of the screening system for cell death-inducing peptides KLLNLISKLF and LNLISKLF that were derived from the MTD of Noxa induce cancer cell death after conjugation with octa-arginine (R8) as a CPP (12,13). We first tested whether these peptides induce cell death in our screening system (22). Consequently, the peptides combined with CPP, e.g., KLLNLISKLF-R8, had cell death-inducing activity (Fig. 1A), whereas KLLNLISKLF and R8 did not show cell death activity (Fig. 1A). LNLISKLF-R8 was shown to have a higher activity compared with KLLNLISKLF-R8. This may be due to the difference in the amount of peptide contained in the medium between two peptides. It is generally believed that shorter peptides have a higher solubility than the longer peptides. Therefore, we assume that a larger amount of LNLISKLF-R8 was released than KLLNLISKLF-R8 into the medium from a peptide-containing disk. Cell morphology was also changed only when the cells were treated with the peptides combined with CPP (Fig. 1B). These results are in agreement with findings of other reports (12,13). Therefore, we hypothesized that the peptides derived from MTD of Noxa could be employed as original peptides for screening for peptides with higher activity in our system.

A screening for peptides with higher cell death-inducing activity by modifying the peptide sequences To obtain peptides with higher activity, the cell death-inducing peptide (LNLISKLF) was modified using our system. We tried alanine scanning to predict the amino acid position that has a potential to yield more active peptides via an amino acid substitution. In our previous report (18), the sequence of cell death-inducing peptides derived from TRAIL was substituted with 20 amino acids. As for substitution with alanine, higher activity after this substitution may help to identify more active peptides by substitutions with other amino acids. On the other hand, at the position that showed lower activity after substitution with alanine, more active peptides were not obtained.

Therefore, in the present study, we substituted each amino acid of the cell death-inducing peptide with alanine (A) and selected the position that showed higher activity after this substitution as a candidate position. As a result, although significant differences were not observed, peptides with asparagine (N) substitution at the second position, serine (S) substitution at the fifth position, or lysine (K) substitution at the sixth position (from the N terminus) showed relatively higher activity after substitution with A (cell viability of LALISKLF-R8 = 53.6% ± 23.4%, cell viability of LNLIAKLF-R8 = 47.2% ± 16.0% and cell viability of LNLISALF-R8 = 74.8% ± 18.7%) than that of the original one (cell viability = 78.2% ± 32.3%; Fig. 2). In the subsequent experiments, we selected LALISKLF-R8 and LNLIAKLF-R8 as the candidates because their cell-death inducing activities were relatively higher than the other peptides.

These positions were substituted with other 19 amino acids to possibly obtain more active peptides. The peptides whose N was substituted with 19 other amino acids showed no significant difference in cell death-inducing activities compared with the original peptide (Fig. 3A). On the other hand, the peptide where we substituted S with phenylalanine (F), valine (V), tryptophan (W), or tyrosine (Y) showed higher activity (cell viability of LNLIFKLF-R8 = 15.3% ± 5.3%, LNLIVKLF-R8 = 15.6% ± 3.6%, LNLIWKLF-R8 = 10.7% ± 1.8%, and LNLIYKLF-R8 14.2% ± 5.1%) than that of the original one (cell viability = 45.0% ± 12.5%; Fig. 3B).

Our screening system can assess intracellular function of many peptides, but we cannot guarantee that the medium contained each peptide at the same concentration because the solubility of peptides synthesized on the cellulose membrane was not the same (22). To test the ability of the modified peptides more precisely, we

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