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

Short title: Effective modification of intracellular peptides

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ABSTRACT

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.

Introduction

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, Kim *et al.* 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 (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 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, Maidstore, UK) was activated using β-alanine as the N-terminal basal spacer. An Fmoc-Photo-Linker (sc-294977A, SANTA CRUZ, Texas, 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

111 150 μL of serum-free Dulbecco's modified Eagle medium (DMEM, 08458-16; Nacalai
112 Tesque, Kyoto, Japan) at 37 °C for 1 h. After release of each peptide, the medium
113 containing the peptide was filtered into a 96-well plate by vacuum filtration (MultiScreen
114 HTS Vacuum Manifold; Merck Millipore) to remove various insoluble materials, and was
115 subsequently used for the assay (22).
116
117 Cell culture
118 MCF-7 cells were maintained in 75 cm^2 flasks (658170; Greiner Bio-One,
119 Frickenhausen, Germany) cultured in DMEM supplemented with 10% fetal bovine serum
120 (FBS, Biosera; Nuaille, France), and 1% Penicillin/Streptomycin (PS, 168-23191; Wako).
121 The cells were cultured in a humidified 5% CO_2 incubator at 37 °C to approximately 80%
122 confluence.
123
124 The cytotoxicity assay
125 MCF-7 cells (10^4 cells/well) were plated in wells of 96-well plates (TR5003; Nippon
126 Genetics Co., Ltd., Tokyo, Japan) and cultured for 24 h. The culture medium was removed,
127 and we added the medium containing peptides. The plate was incubated for 3 h in the
128 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, F10471, Massachusetts, 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 Image J 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

183 (S) substitution at the fifth position, or lysine (K) substitution at the sixth position (from
184 the N terminus) showed relatively higher activity after substitution with A (cell viability
185 of LALISKLF-R8 = 53.6% ± 23.4%, cell viability of LNLIAKLF-R8 = 47.2% ± 16.0%
186 and cell viability of LNLISALF-R8 = 75.4% ± 17.3%) than that of the original one (cell
187 viability = 78.2% ± 32.3%; Fig. 2). In the subsequent experiments, we selected
188 LALISKLF-R8 and LNLIAKLF-R8 as the candidates because their cell-death inducing
189 activities were relatively higher than the other peptides.

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

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

test the ability of the modified peptides more precisely, we purchased purified synthetic peptides. We prepared a medium containing a peptide at the same concentration and then we checked cell death activity. The activity was almost the same as in the results listed above (data not shown). Next, we evaluated the dose dependence of the peptide's effects. The peptide was dissolved in the medium at the concentrations of 5, 10, 25, 50, 75, and 100 μmol/L, and we assessed cell death-inducing activity. As a result, all modified peptides selected by our system showed significantly higher activities than that of the original one (Fig. 4). The concentration of the original peptide (LNLISKLF-R8) that caused 50% cell death was 44.9 μmol/L. On the other hand, the concentration of the modified peptides was remarkably low: 14.4 μmol/L for LNLIFKLF-R8, 22.1 μmol/L for LNLIVKLF-R8, 10.1 μmol/L for LNLIWKLF-R8, and 17.3 μmol/L for LNLIYKLF-R8. Thus, the peptides selected by our screening system showed higher cell death-inducing activity. These results suggest that the intracellular activity of peptides can be assessed using the proposed screening system. This suggests that the proposed screening system has a potential for identifying modified peptides with higher activity.

Verification of the mechanism of action of the modified peptide

Finally, we investigated the mechanisms of action of the obtained peptides. The

original peptide (LNLISKLF-R8) has a function of opening the mPT pore, which induces a calcium release from mitochondria (12). We assessed whether the more active peptides (LNLIWKLF-R8) induce a calcium release as the original peptide does. The original and modified peptides were dissolved in the medium at the concentration capable to induce 50% cell death (44.9 μmol/L for LNLISKLF-R8 and 10.1 μmol/L for LNLIWKLF-R8). The changes of calcium concentration in the cytosol were monitored in MCF-7 cells by means of a fluorescent calcium indicator.

As a result, for the original peptide, the cytosolic calcium concentration increased at 60–90 s after peptide treatment as reported by Seo *et al.* (12) (Fig. 5A). On the other hand, for the modified peptide, cytosolic calcium concentration increased at 50–60 s after the peptide treatment (Fig. 5B). These results indicated that both the original and modified peptide induced an increase of intracellular calcium concentration. Therefore, the function of the original peptide (mPT pore opening) may be preserved after modification of the peptide sequence.

As shown in Fig. 5, the calcium level increased more sharply for the modified peptide than for the original one. The difference in the increase in fluorescence was possibly caused by a difference in the interaction between peptides and the target molecule that opens the mPT pore complex (it has not been identified). As for the substituted amino

237 acids that yielded higher activity, F and V are classified as hydrophobic amino acids,
238 whereas F, W, and Y have an aromatic side chain. Hydrophobic amino acids contribute to
239 hydrophobic interactions, and aromatic amino acids contribute to π-stacking interactions.
240 Because these interactions are the main factors of protein–protein interactions (23,24),
241 they are expected to be a major factor in the interaction between the peptides and target
242 molecule, with which the original peptide may bind. Thus, the modified peptides with
243 substitution of S with F, V, W, or Y may have a stronger hydrophobic interaction and π-
244 stacking interaction with the target molecule than the original peptide does. On the other
245 hand, modified peptides whose N was substituted by F, V, W, or Y did not show a higher
246 activity. The peptide structure of LNLISKLF is predicted to be alpha-helical by the helical
247 wheel plot. N is plotted at the center of the hydrophilic core; thus, the peptide structure
248 may be changed by substituting N with F, V, W, or Y. The structural change may lower
249 the cell death-inducing activity of peptides. As for the substitution of S, it is plotted near
250 the hydrophobic core; thus, the peptide structure may not be changed.

251 In conclusion, we obtained 4 peptides with higher activity by means of a cell death-
252 inducing peptide using the screening system for intracellular functional peptides. Higher
253 activity of the cell death induction by the modified peptides may be caused by increased
254 hydrophobicity of the peptides 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, and peptides with a higher activity can be obtained via an amino acid substitution. The proposed screening system can be used for identifying intracellular functional peptides with higher activity through comprehensive screening.

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Figure legends

Figure 1 Validation of the screening system for cell death-inducing peptides identified from Noxa.

MCF-7 cells were incubated with cell death-inducing peptides (KLLNLISKLF-R8 and LNLISKLF-R8), KLLNLISKLF, or R8. (A) After treatment with these peptides for 3 h, cell death was assayed by means of the Cell Counting Kit-8. Cell viability results from the group without a peptide were set to 100%. Each peptide spot was placed into a well and used for one spot/well analysis. (B) Bright field images were obtained by means of a microscope before treatment with a peptide and 3 h after treatment. Approximately 5 ×

381 10^4 MCF-7 cells were seeded in a glass base dish 24 h before the experiment. Scale bar
382 = 50 μm. **p < 0.01 versus no-peptide group.
383
384 Figure 2 Prediction for the amino acids substitution site to obtain peptides with higher
385 activity by alanine scanning.
386 Each amino acid of the peptide sequence was substituted with alanine. MCF-7 cells
387 were treated with each modified peptide. After treatment with these peptides for 3 h, cell
388 death was assayed by means of the Cell Counting Kit-8. Cell viability results from the
389 group without a peptide were set to 100%. Each peptide spot was placed into a well (one
390 spot/well). After the release, the medium containing peptide was diluted fourfold by
391 adding serum-free DMEM and was used for an assay.
392
393 Figure 3 Substitution analysis based on the LNLISKLF peptide, using our screening
394 system.
395 MCF-7 cells were treated with each modified peptide. (A) Asparagine (N) at the second
396 position was substituted with other 19 amino acids and (B) serine (S) at the fifth position
397 was substituted with other 19 amino acids. After treatment with these peptides for 3 h,
398 cell death was assayed by means of the Cell Counting Kit-8. Cell viability results from

399 the group without a peptide were set to 100%. Each peptide spot was placed into a well
400 (one spot/well). After the release, the medium containing peptide was diluted fourfold by
401 adding serum-free DMEM and was used for an assay. *$p < 0.05$ versus LNLISKLF-R8
402 treatment.
403
404 Figure 4 Comparison of the cell death activity of the synthesized LNLISKLF-R8 and
405 identification of peptides more active at the same concentration.
406 MCF-7 cells were incubated with various concentration of LNLISKLF-R8 and more
407 active peptides (LNLIFKLF-R8, LNLIVKLF-R8, LNLIWKLF-R8, and LNLIYKLF-R8).
408 After treatment with these peptides for 3 h, cell death was assayed by means of the Cell
409 Counting Kit-8. Cell viability results from the group without a peptide were set to 100%.
410 Image J software was used for curve fitting of each experimental dataset.
411
412 Figure 5 Comparison of the cell death-inducing pathway of the basic peptide and a more
413 active peptide.
414 Cell death-inducing peptides induce changes of the intracellular calcium level. MCF-7
415 cells were loaded with a Fluo-4-direct™ solution for 30 min and then were treated with
416 (A) 44.9 μmol/L LNLISKLF-R8, (B) 10.1 μmol/L LNLIWKLF-R8, or (C) only serum

free DMEM. Fluorescein images were obtained by means of a microscope with a spinning-disk confocal system at 10-s intervals for 5 min. Relative fluorescein intensities (F/F0) were calculated for 10 cells as mean of fluorescein intensity changes from fluorescein intensity at time 0. The Image J software was used for measuring fluorescein intensity.