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Cell Penetrating Penta-Peptides (CPP5s) and Bax Inhibiting Peptides (BIPs): Protocol for their application

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Abstract

The first series of Cell Penetrating Penta-peptides (CPP5s) were discovered as cytoprotective penta-peptides designed from the Bax inhibiting domain of Ku70. Bax is an inducer of programmed cell death and Ku70 is a multifunctional protein maintaining genomic stability and protecting cells from death by inhibiting the cytotoxic activity of Bax. Since these peptides bind and inhibit Bax, they are named Bax Inhibiting Peptides (BIPs). The second series of CPP5s were developed by mutating BIP's amino acid sequences to abolish the Bax binding activity. These peptides were used as negative control peptides to evaluate the Bax inhibiting activity of BIPs. CPP5s are able to enter cells when they are added to the culture medium. The mechanism of cell entry of CPP5s is not yet understood. Numerous studies showed that BIP rescued cells from cytotoxic stresses both in cell culture and animal model, suggesting the therapeutic potential of BIP. Both BIPs and non-cytoprotective CPP5s did not show significant toxicity even at 1.6 mM concentration in cell culture. Our recent study suggests that CPP5s has the protein transduction activity, though only Green Fluorescent Protein (GFP) has been tested as a cargo protein. If CPP5s can deliver wide range of cargo molecules into the cell, CPP5s may be utilized as non-toxic drug delivery tool. In this article, we describe our laboratory's protocols of how to synthesize, store, and apply CPP5s for the examination of their activities of cell penetration and cytoprotection.

Keywords

Cell Penetrating Peptide; Cell Penetrating Penta-peptide; Ku70; Bax; Protein Transduction; Apoptosis; Programmed Cell Death

1. Introduction

The first series of cell penetrating penta-peptides (CPP5s) were discovered as Bax Inhibiting Peptides (BIPs) that were designed from the Bax binding domain of Ku70 (1–3). Ku70 is a multifunction protein involved in non-homologous end joining DNA repair (4, 5) and apoptosis regulation (1, 2). Ku70 binds and inhibits the proapoptotic protein Bax in the cytosol and thus Ku70 protects cells from Bax-mediated cell death (1, 2). We found the Bax binding domain comprised of five amino acids in the C-terminus of Ku70 (1, 3). Interestingly, synthetic penta-peptides of this domain showed cell penetrating activity and these peptides were able to rescue cells from death by simply adding them into the cell culture (1). These cytoprotective peptides were named BIPs. Various versions of BIPs were designed from the Bax binding domain of Ku70 of different species including human (VPMLK), rat (VPALR), and mouse (VPTLK) (1, 3). In addition, the second series of

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CPP5s (e.g. IPMIK and KLPVM) were developed by mutating the BIP sequence. These non-cytoprotective CPP5s were originally designed as negative control peptides to verify the Bax inhibiting activity of Ku70-derived BIPs. In this article, we describe the experimental protocols of how to determine the activities of cell penetration and cytoprotection.

2. Materials and Methods

2.1 Peptides Synthesis

All the peptides examined in our laboratory were synthesized by companies (for example, Biopeptide Co., Inc. San Diego CA, USA) as order-made peptides. Since we often use CPP5s for the protection of cells from damage, we purchase HPLC purified peptide (98%< purity). It is reported that the usage of D-type amino acids increases the stability of peptides in the cell since D-type peptides are resistant to protease-dependent degradation(6). We compared the cytoprotective activities of D-type and L-type VPTLK (BIP designed from mouse Ku70) in HeLa cells, however, we could not see a significant difference between these two versions (unpublished observation by Jose Gomez and Shigemi Matsuyama). Therefore, at least in the case of VPTLK, the usage of D-type amino acid is not effective to increase the biological activity. We have not examined D-type amino acids in other CPP5s. It is still possible that the utilization of D-type amino acids improves the biological activities in other CPP5s.

2.2 Storage of the Peptides

When we order CPP5 synthesis, we request companies to dispense peptide in tubes with known quantity, for example, 5 mg (dried powder) in each tube. Upon arrival, the peptides are stored in -80°C or -20°C freezer. As long as peptides are stored as dried powder, we did not observe significant decrease of biological activities after one year or longer storage in the freezer.

For preparation of stock solution, we use dimethyl sulfoxide (DMSO) to dissolve peptides at 100 or 200 mM concentration in polypropylene tube (0.5 ml tube) and the solutions are stored at -20°C or -80°C . The stock solution prepared by DMSO showed stable biological activities (cell penetration and cytoprotection) even after three times of freeze-thaw cycle. It is very important to use non-oxidized DMSO which has been stored by filling the container with non-reactive gas (e.g. N_2 gas). It is not recommended to use old DMSO stored at room temperature without filling the bottle by non-reactive gas. Our laboratory uses DMSO dispensed in small ample tubes (5 ml for each ample) (Sigma-Aldrich, D2650). To be noted, it is not recommended to use glass tubes for the preparation of CPP5 stock solution if DMSO is used. Once we experienced the disappearance of cytoprotective activity of VPMLK by dissolving the peptide in DMSO using glass tube, though we do not know the exact reason for this problem. After this experience, we are using polypropylene tube (0.5 ml or 1.5 ml centrifuge tube) for storage and preparation of stock solution.

Most of CPP5s are water soluble and it is possible to prepare stock solution in water, phosphate buffer saline (PBS), or other types of buffer. For example, stock solution in PBS may be suitable for *in vivo* experiment. We examined the effects of freeze-thaw cycle on cytoprotective activities of VPTLK and VPMLK dissolved in PBS. Two times of freeze-thaw cycle did not show significant decrease of the activity, but the decrease was observed after the third cycle. Therefore, it is recommended to avoid more than three times of freeze-thaw cycle when stock solution is prepared in water or buffer.

2.3 List of materials needed for experiments introduced in this chapter

- 6 well culture dish.

- 10% Fetal Calf Serum (FCS) containing Dulbecco's' Modified Minimum Essential Medium (DMEM).
- FITC (or other appropriate florescent dye) conjugated-CPP5
- BIP (fluorescent dye conjugation is not necessary to the inhibition of Bax activity).
- 0.25% Trypsin-EDTA
- Hank's Buffered Saline Solution (HBSS)
- Hoechst Dye (33258 or the similar nuclear staining dye)

3. Methods

3.1 General Information

Addition of peptide to cell culture—In general, we add CPP5 into the culture medium at the concentration ranging from 10 μ M - 400 μ M. In most of the cell types, the cell entry of FITC-CPP5 can be detected by fluorescent microscope by culturing cells in the presence of 10 μ M or above for more than 3 hrs. In the case of HeLa cells and DAMI cells (human megakaryotic cell line), the cell entry of FITC-VPTLK and -KLPVM was observed within 15 min of the incubation when FITC's fluorescence was measured by flow cytometry (1, 7). To protect cells from apoptosis by BIP, 10–400 μ M BIP is required depending on cell type and strength of stress. For example, 200 μ M is required to protect HEK293T cells from Bax over-expression (transient transfection of Bax expressing plasmid).

CPP5 is stable in culture medium for more than 3 days, since BIP maintained its cytoprotective activity in cell culture for 3 days after the addition of BIP to the media. In our laboratory, we usually re-add BIP every three days (by replacing old medium with fresh medium) if we need to inhibit Bax-mediated cell death for longer than 3 days.

Detection of Fluorescence dye-labeled CPP5 in the cell—For the detection of FITC-labeled CPP5 in the cell, we usually incubate cells for 3 hrs in the presence of the peptide at the concentration of 10 μ M or higher. The presence of serum in the medium does not interfere the cellular uptake of CPP5. Therefore, commonly used culture media containing 10% serum can be used for the examination of the cell entry and cytoprotection by CPP5.

Incubation time can be varied depends on cell type and CPP5 concentration. In the case of HeLa and DAMI cells, 15 min incubation was sufficient to detect the cell penetrating activity of FITC-labeled CPP5 (100 μ M concentration in the medium) by flow cytometric analysis (1, 7).

Cell death inhibition by BIP—As described earlier, BIPs was designed from Bax inhibiting domain of Ku70 from several species(1, 3). VPMLK and PMLKE were designed from human Ku70, VPTLK was from mouse Ku70, VPALR was from rat Ku70 (3). All these BIPs were able to bind human Bax, and they can rescue human cells from Bax-mediated cell death. In our laboratory, VPTLK is mainly used since this BIP showed the best stability for cytoprotection after the long storage for more than one year. We experienced a significant decrease of Bax inhibiting activity of VPMLK and PMLKE after several freeze-thaw cycles. At present, we do not know the exact reason of the loss of activity, but we suspect that oxidation of methionine (M) during the long storage might be the cause of the problem. On the other hand, VPTLK showed very constant cytoprotective activity. If there is no special reason to use VPMLK or PLMKE, it is recommended to use VPTLK for the protection of cells from Bax-mediated cell death.

In general, we use 50–400 μ M BIP to protect cells from stresses that activate Bax (1–3). The concentration of effective BIP should be determined by dose-dependent analysis. For example, 200 μ M BIP is effective to protect HeLa cells, human epithelial kidney (HEK) 293T and HEK293 cells, DAMI cells (human megakaryocytic cell line), human umbilical cord endothelial cells (HUVEC), and mouse embryonic fibroblasts (MEFs) from several stresses including anti-cancer drugs (etoposide, doxorubicin, taxol, etc) and other chemicals inducing Bax-mediated apoptosis (e.g. staurosporin) (1–3). There are numerous reports showing the protection of various cell types from cytotoxic stresses. For example, VPMLK and VPTLK protected retinal cells from oxidative stress and glutamate-induced cell death (8, 9), and stresses inducing macular degeneration (10). BIP also protected neuron from trophic factor deprivation (11) and poly-glutamate overexpression (2). It was also shown that BIP increased the survival of transplanted liver cells in mouse model by the pre-incubation of liver cells in BIP containing medium (12). To be noted, VPMLK was able to protect the protozoan parasite (*Giardia lamblia*) from death caused by ectopic expression of mammalian Bax protein (13).

Addition of peptides to animal model—There are some reports examining the effects of BIP in mouse model (11, 12, 14), and these reports indicate that BIP has a therapeutic potential to rescue damaged cells from Bax-mediated cell demise program. For example, BIP was able to rescue retinal cells from apoptosis induced by optic nerve injury in mouse (14). In this case, four microliters of BIP solution (46.9 μ g/ μ l in phosphate buffered saline) was intravitreally injected the eye for every 3 days for 6 days.

In our laboratory, we examined the toxicity of KLPVM and VPTLK in mouse by injecting these peptides by i.v., i.p., and s.c. We examined maximum dose of 275 mg/kg, but we did not observe any toxicity and the mice were able to show normal reproductive activity after the injection of the peptides (unpublished observation by Jose Gomez and Shigemi Matsuyama).

Examination of Protein Transduction activity—In addition to cell penetrating activity, there is a possibility that CPP5 has protein transduction activity. Among several CPP5s, we tested KLPVM and VPTLK whether these CPP5s can deliver Green Fluorescence Protein (GFP) into cultured cells (1, 7). CPP5 sequence was fused to the C-terminus of GFP and the recombinant proteins of GFP-CPP5 were prepared. HeLa cells were cultured with 1 μ M of GFP-VPTLK, GFP-KLPVM, or GFP (no CPP5 tag) for 24 hrs in 10% FCS containing DMEM medium. After the extensive wash of the cells by culture medium, cells were observed under the fluorescent microscope. As reported in our previous publication (1, 7), green fluorescence was observed from cells incubated with GFP-VPTLK and GFP-KLPVM, but not by GFP. This result suggests that CPP5 has a potential to deliver cargo molecule across the plasma membrane. At present, we are performing further experiments to determine whether CPP5 has protein transduction as TAT peptide does (15).

3. 2. Protocol Examples

3.2.1. Experiment 1—Examination of cell penetration activity of FITC-labeled CPP5 in HeLa cells.

1. Start cell culture in 6 well dish (1×10^5 cells/2ml/well). Use 10% FCS containing DMEM. Incubate cells for overnight. **See NOTE section.**
2. Prepare FITC-CPP5 containing medium. For example, if you use 100 mM stock solution prepared in DMSO, add 10 μ l of this stock solution into 10 ml DMEM containing 10% FCS to prepare 100 μ M FITC-CPP5 containing medium.
3. Aspirate the medium from each well and add FITC-CPP5 containing medium.

4. Incubate 3 hrs.
5. Wash cells by HBSS at least 2 times.
6. Re-add DMEM containing 10% FCS, and observe cells under fluorescent microscope. If flow cytometric analysis is performed, collect cells by trypsinization (use 0.25% trypsin EDTA) and wash cells by HBSS at least one time by centrifugation. Then, re-suspend cells in HBSS.

3.2.2. Experiment 2—Protection of HeLa cells from cytotoxic stresses by BIP.

1. Start cell culture in 6 well dish (1×10^5 cells/2ml/well). *See NOTE section*
2. Prepare BIP containing medium. For example, if you use 100 mM stock solution prepared in DMSO, add 20 μ l of this stock solution into 10 ml DMEM containing 10% FCS to prepare 200 μ M BIP containing medium.
3. Change the medium with BIP containing medium, and pre-incubate cells for 3 hrs or overnight.
4. Treat cells with apoptotic stresses. For example, add staurosporin (final concentration 100 nM), Etoposide (1–10 μ M), or Doxorubicine (1 μ M). If medium change is necessary, do not forget to re-add BIP into the fresh medium.
5. Incubate cells with apoptosis-inducing reagent for the necessary period such as 12 hrs, 24hrs, and 48 hrs.
6. Add Hoechst dye to the medium at the final concentration of 4 μ g/ml. Incubate cells at least for 10 min.
7. Observe cells under the fluorescent microscope and detect apoptosis by the change of nuclear morphology (nuclear condensation and nuclear fragmentation).

4. NOTE (please insert this “Note” at the end of “3. Method” section)

Cell density is an important factor

The efficiency of cell penetration by CPP5s can be significantly influenced by culture condition, especially by cell density (cell number per dish). In “100% confluent” condition, the efficiency of cell entry of CPP5s becomes less than that in lower cell density condition. Important issue is that cell density must be accurately controlled in each experiment to obtain constant result. In this protocol, HeLa cells are plated at the density of 1×10^5 cells/well that results in 30–40% confluent condition. In this condition, CPP5s show very fast cell penetration activity (cell entry can be detected within 15 min of incubation).

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