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Structural Development of Cell-Penetrating Peptides Containing Cationic Proline Derivatives

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We designed and synthesized a series of cell-penetrating peptides containing cationic proline derivatives (Pro4SGu) that exhibited responsive changes in their secondary structures to the cellular environment. Effects of the peptide length and steric arrangement of the side chain in cationic proline derivatives [Pro4SGu and Pro4RGu] on their secondary structures and cell membrane permeability were investigated. Moreover, peptides 3 and 8 exhibited efficient intracellular delivery of plasmid DNA.

Key words cell-penetrating peptide; cationic proline derivative; cellular environment; drug delivery system (DDS) carrier

Cell penetrating peptides (CPPs) are useful tools in delivering hydrophilic cargo molecules such as proteins and nucleic acids into cells. It is well known that human immunodeficiency virus (HIV)-1 Tat and oligoarginine (Rn), which are representative CPPs contain cationic amino acids.1,2) Recently, it has been reported that the CPPs exert their cell membrane permeability based on the interaction between the side-chain guanidino groups of arginine and acidic groups existing on the cell surface.3,4) However, the influences of the secondary structure of CPPs on their cell membrane permeability has not been fully elucidated. To date, several methods to control peptide secondary structures have been developed. The introduction of unnatural amino acids, such as α,α-disubstituted amino acids (dAAs) or cyclized β-amino acids have been shown to enhance peptide helical structures.5–7) To investigate the effects of the secondary structure of CPPs on their cell membrane permeability, we designed and synthesized novel CPP derivatives containing dAAs residues such as α-amino-isobutyric acid (Aib).5) For example, the nonapeptides FAM-βAla-(l-Arg-l-Arg-Aib)3-NH2 (1, FAM: 6-fluorescein), FAM-βAla-(l-Arg-d-Arg-Aib)3-NH2 (2) and FAM-βAla-(d-Arg-d-Arg-Aib)3-NH2 (ent-1) were synthesized, and their cell-penetrating ability were evaluated. The studies revealed that the peptides 1 and ent-1, which formed a right-handed (P) and a left-handed (M) helical structure, respectively, showed higher cell-penetrating ability than peptide 2, which formed a random structure. These results demonstrated that formation of a helical structure is favorable to exert the cell permeability of Arg based CPPs. Furthermore, we reported that peptides containing cationic cyclic dAA residue ApiC2Gu [FAM-βAla-(l-Arg-l-Arg-ApIC2Gu)3-NH2] formed a stable helical structure and efficiently delivered plasmid DNA (pDNA) into cells.9) Moreover, we also reported that a series of CPPs containing the cationic proline derivative FAM-βAla-(l-Arg-l-Arg-Pro4SGu)3-NH2 (3, Pro4SGu: a proline derivative containing a guanidine group at the 4-position) efficiently delivered pDNA into cells, with secondary structure of the peptide changing in response to the environment.10) Although peptide 3 formed a random structure in phosphate buffered saline (PBS) buffer, it formed a helical structure in 1% sodium dodecyl sulfate (SDS) PBS buffer, which mimicked the environment as near the cell membrane (Fig. 1).

To further investigate the effects of the secondary structure on their cell membrane permeability, we designed and synthe-

Fig. 1. Chemical Structures of Homochiral (1, ent-1 and 3), Heterochiral Peptides (2) and Pro4SGu

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sized a set of peptide 3 derivatives (4, 5), which were composed of a different ratio of L-Arg and d-Arg, and shortened peptides (6, 7). It has also been reported that the chirality of the amino acid side-chain in a peptide sequence can affect its secondary structure.11,12) Therefore, we also synthesized peptide 8 containing Pro 4RGu and investigated the effects of the chirality at the side chain of proline residues on their secondary structure and cell-membrane permeability (Fig. 2). Moreover, to demonstrate the utility of the synthesized peptides, the pDNA transfection assay was performed.

Results and Discussion

The Pro 4RGu was prepared as previously reported (Chart 1).13) The amino and carboxyl groups of (2S,4R)-hydroxyproline were protected with carboxybenzyl (Cbz) and benzyl groups, respectively. The hydroxy group of 9 was converted to the azide group by the Mitsunobu reaction to yield 10. After the reduction of azide group by Staudinger reaction, the amino group was guanidinylated. Subsequently, the Cbz and benzyl groups were deprotected, and the resulting amino group was protected with fluorenylmethyloxycarbonyl (Fmoc) group to obtain the target compound Pro 4RGu.

The designed peptides were synthesized using solid-phase methods, purified by reverse-phase HPLC, and were characterized by LC-MS. The preferred secondary structures of synthesized peptides 3–8 were analyzed based on their circular dichroism (CD) spectra, shown in Fig. 3 (peptide concentration of 100 μM in PBS buffer containing 1% SDS). All peptides formed random structures in PBS buffer (Figs. 3b, d, f). However, the homochiral L-peptides 3 and 7 displayed negative maxima at approximately 208 and 222 nm in PBS buffer containing 1% SDS (Fig. 3c), whereas d-Arg based peptide 5 showed positive maxima at approximately 208 and 222 nm (Fig. 3a). These results indicated that L-Arg based peptides 3 and 7 and D-Arg based peptide 5 possessed a right-handed (P) and a left-handed (M) screw sense, respectively.11,14) In addition, these results revealed that the chirality at the side chain of the proline residues did not affect its secondary structure (Fig. 3e). Moreover, the shortened peptides 6 and heterochiral peptide 4 formed random structures both in PBS buffer with and without 1% SDS (Figs. 3a, c). These data demonstrated that a peptide length longer than six amino acid residues and homochirality were required to change their secondary structures from a random to helical structure depending on the environments.

Next, we evaluated the cell membrane permeability of the synthesized peptides against adhesive HeLa cells. The cells were treated with 1 μM synthesized peptides 3–8 at 37°C for 2h, and the intracellular uptake was analyzed by flow cytometry. Cellular uptakes of the tested peptides were represented as the ratio to that of R9 [FAM-βAla-(Arg)9-NH2]. As shown in Fig. 4, L- and d-based peptides 4 and 5 showed the lower intracellular uptake compared to L-based peptide 3, and the shortened peptides 6 and 7 showed mild intracellular uptake. These data indicated that the peptide helicity and length played an important role in permeating the cells. In addition, peptides 3 and 8 exhibited the same extent of intracellular uptake, demonstrating that the chirality of the side chains on the proline residues did not affect their intracellular uptake.

Next, we assessed the internalization mechanisms of pep-
tides 8 by using HeLa cells. It has been previously reported that peptide 3 internalized into the cells via macropinocytosis. Therefore, in order to confirm the internalization pathway of peptide 8, the cells were incubated with the tested peptides in the presence of several endocytosis inhibitors (amiloride, an inhibitor of macropinocytosis; nystatin, an inhibitor of caveolae-mediated endocytosis; and sucrose, an inhibitor of clathrin-mediated endocytosis), and the intracellular uptake was measured by flow cytometry (Fig. 5a). As a result, the intracellular uptake of peptide 8 was inhibited by...
treatment with amiloride, whereas nystatin and sucrose exhibited mild effects on intracellular uptake of peptide 8. Moreover, the intracellular distribution of peptides 3, 8, and R9 in HeLa cells was investigated by confocal laser scanning microscopy (CLSM). HeLa cells were treated with 10µM peptide solution. After incubation for 2h at 37°C, nuclei were stained with Hoechst 33342. As shown in Fig. 5b, R9 was observed as small green spots, whereas peptides 3 and 8 were widely distributed in the cytosol. We speculate that the peptide 8 internalized into cells via not only macropinocytosis, but also direct penetration of cell membrane, which is similar to that observed for peptide 3.

Next, we performed the cell viability assay using a cell counting kit-8 following the manufacturer’s protocol. It has been previously reported that R9 does not exhibit cytotoxicity against several cell lines.9,10) The results of the cytotoxicity analysis are shown in Fig. 6. The HeLa cells were treated with peptides 3–8 at concentrations of 1, 4, and 8µM. None of the peptides exhibited the significant cytotoxicity.

Finally, we performed a pDNA intracellular delivery experiment using the synthesized peptides 3–8. The peptide-pDNA complexes were prepared at a 4:1 charge ratio, because the residual molar ratio of the amino and/or guanidine groups in the peptide corresponds to the number of phosphate groups in the pDNA. Huh-7 cells were incubated with the peptide-pDNA complexes, encoding luciferase, complex and the delivery efficiency was assessed by chemiluminescence. As shown in Fig. 7, peptides 3 and 8 exhibited higher transfection efficiency than R9, and they exhibited the same extent of intracellular delivery efficiency with jetPEI, which is a commercially available transfection reagent. However, peptides 6 and 7 did not exhibit intracellular uptake. Although heterochiral peptides 4 and 5 exhibited a lower intracellular uptake compared to homochiral peptides 3 and 8, they exerted the potent intracellular delivery efficiency. It has been reported that the introduction of D-amino acid residues would improve their stability against the degrading enzymes.19) Therefore, heterochiral peptides 4 and 5 may exert continuous pDNA delivery, and exhibit the same extent of transfection efficacy compared to homochiral peptides 3 and 8.

Conclusion

In conclusion, we designed and synthesized a series of homochiral and heterochiral CPP derivatives containing cationic proline derivatives Pro4SGu and Pro4RGu, and evaluated their cell membrane permeability against HeLa cells. It was demonstrated that peptide 8 showed responsive changes in the secondary structure depending on its environment, with the same extent of cell membrane permeability as peptide 3. Moreover, these results revealed that the chirality at the side chain of proline residues did not affect their secondary struc-
the peptides containing cationic proline derivatives could be functioned as a carrier of pDNA. These results indicated that...  

Experimental

General All coupling reagents were obtained from Watanabe Chemical Industries, Ltd. (Japan) and were used as supplied without further purification. Fmoc-protected amino acids were obtained from Tokyo Chemical Industry Co., Ltd. (Japan) and Watanabe Chemical Industries, Ltd. Analytical TLC was performed on Silica Gel F 254. High-resolution Merck (Japan) and Watanabe Chemical Industries, Ltd. were supplied without further purification. Fmoc-protected amino acids were obtained from Tokyo Chemical Industry Co., Ltd. (Japan) and were used as solvents. Peptide concentration; 100 µM. After the peptide elongation, the resin was suspended in cleavage cocktail [1.9 mL trifluoroacetic acid (TFA), 50 µL water, 50 µL triisopropylsilane; final concentration: 95% TFA, 2.5% water, 2.5% triisopropylsilane] for 3 h at room temperature. The TFA solution was evaporated to a small volume under a stream of N₂ and dripped into cold ether to precipitate the peptides. The dried crude peptides were dissolved in 1.3 mL of 50% acetonitrile in water and then purified by reversed-phase HPLC using a Discovery® BIO Wide Pore C18 column (25 cm×21.2 mm). After being purified, the peptide solutions were lyophilized. Peptide purity was assessed using analytical HPLC and a Discovery® BIO Wide Pore C18 column (25 cm×4.6 mm; solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 1.0 mL·mL⁻¹, gradient: 10–100% gradient of solvent B over 30 min). The peptides were characterized by LCMS-IT-TOF spectroscopy.

CD Spectrometry CD spectra were recorded with a Jasco J-720W spectropolarimeter using a 1.0 mm path length cell. The data are expressed in terms of [θ]; i.e., total molar ellipticity (deg cm² dmol⁻¹). 20 mM phosphate buffer (pH=7.4) and 1% SDS in 20 mM phosphate buffer (pH=7.4) were used as solvents. Peptide concentration; 100 µM.

Cell Culture HeLa cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). HeLa cells was grown on 10-mm dishes and incubated at 37°C under 5% CO₂ humidified atmosphere.

Flow Cytometry HeLa cells were seeded in 6-well dishes at a density of 2.0×10⁵ cells/well and cultured in DMEM for 24 h, respectively. The cells were treated with each peptide (peptide concentration; 1 µM) and incubated for 2 h. Then, the cells were washed three times with PBS supplemented with heparin (20 units/mL) and detached by treatment of trypsin–ethylenediaminetetraacetic acid (EDTA). The collected cells were pelleted by centrifugation at 3000 rpm for 5 min and the supernatant was removed. The cells were washed twice with PBS buffer. Then, the collected cells were suspended in 500 µL PBS containing 3% FBS and mean fluorescence intensity in cells was measured by flow cytometer.

Cytotoxicity HeLa cells were seeded onto 96-well culture plate (5000 cells/well) and incubated for 24 h in DMEM containing 10% FBS. The medium was replaced and peptide solution in fresh DMEM was added at each concentration (1, 4, 8 µM). After 24 h, cell viability was evaluated using cell counting kit-8 (DOJINDO, Japan) following to the manufacturer’s protocol. The results are presented as the mean and standard deviation values obtained from 3 samples.
Inhibition of Endocytosis  The cells were seeded onto 6-well culture plates (400000 cells/well) and incubated overnight in 2 mL of DMEM containing 10% FBS. After the medium had been replaced with fresh medium containing 10% FBS in the absence or presence of amiloride (5 mM), sucrose (0.4 M), or nystatin (25 μg/mL), the cells were pre-incubated at 37°C for 30 min. Peptide solution was applied to each well at a concentration of 1 μM. After the cells had been incubated for 1 h, the medium was removed, and the cells were washed 3 times with PBS supplemented with heparin (20 units/mL) and detached by treatment of trypsin–EDTA. Then, fluorescence intensity in the cells was measured as above. The results are presented as the mean and standard deviation values obtained from 3 samples.

Fluorescence Microscope  HeLa cells were seeded onto glass bottom dish (Matsunami, Osaka, Japan) (100000 cells/well) and incubated overnight in 2 mL of DMEM containing 10% FBS. The medium was then replaced with fresh medium containing 10% FBS, and peptide solution was applied to well at a concentration of 10 μM. After the cells had been incubated for 2 h, the medium was removed, and the cells were washed 3 times with ice-cold PBS supplemented with heparin (20 units/mL). The intracellular distribution of the complexes was observed by CLSM after staining late endosomes/lysosomes with LysoTracer Red and nuclei with Hoechst 33342. CLSM observations were performed using a BZ-9000 (Keyence, Osaka, Japan) equipped with a 40× objective lens.

Direct Cell Permeability by Flow Cytometry Analysis at 37°C  The cells were seeded onto 6-well culture plates (200000 cells/well) and incubated overnight in 2 mL of DMEM containing 10% FBS. After the medium had been replaced with fresh medium containing 10% FBS and peptide solution was applied to each well at a concentration of 1 μM. After the cells had been incubated for 1 h at 37°C, the medium was removed, and the cells were washed 3 times with PBS supplemented with heparin (20 units/mL) and detached by treatment of trypsin–EDTA. Then, fluorescence intensity in the cells was measured as above.

Intracellular Delivery of plasmid DNA  Huh-7 cells were separately seeded onto 24-well culture plates (10000 cells/well) and incubated overnight in 400 μL of DMEM containing 10% FBS. The medium was changed, and the peptide/pDNA complex solutions (33.3 μg pDNA/mL) prepared at charge ratio 4:1, and JetPEI/pDNA (at charge ratio 5:1) were applied to each well. The amount of pDNA was adjusted to 1 μg per well. After 24-h incubation, the medium was replaced with 400 μL of fresh medium, followed by incubation. Luciferase gene expression was then evaluated based on photoluminescence intensity using the Luciferase assay kit and a Lumino-meter (Gene Light GL-210A, Microtec. Co., Ltd., Chiba, Japan). The amount of protein in each well was concommitantly determined using a Micro BCA protein assay kit. The results are presented as the mean and standard deviation obtained from 4 samples.

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