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A novel 11-residual peptaibol-derived carrier peptide for in vitro oligodeoxynucleotide delivery into cell

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Abstract—Using a pore- and channel-forming peptide, TV-XIIa, which is an 11-residual peptaibol isolated from the fungus *Trichoderma viride*, we developed a vehicle for the cellular delivery of such polar biologically active agents as antisense oligodeoxynucleotides (ODNs). To function as an ODN carrier, basic amino acids, 10-mer of lysine, were conjugated to the C-terminus of TV-XIIa and the designed carrier peptide, Ac-U-N-I-I-U-P-L-L-U-P-I-K-K-K-K-K-K-K-K-K-K-OH (U: α-aminoisobutyric acid), was synthesized by the Fmoc-based solid-phase method. The complex between the carrier peptide and ODNs, which was electrostatically formed, was capable of crossing the membranes of NIH3T3 cells and the ODNs were accumulated in the cytoplasm and the nucleus. However, the complex was not taken up by A549 cells. The translocation of the complex occurred at both 4 and 37 °C in NIH3T3 cells and did not seem to involve an energy-dependent endocytic process.

Antisense oligodeoxynucleotides (ODNs) regulate gene expression by binding to a complementary mRNA strand via conventional Watson–Crick hydrogen bonds, thereby providing specific targeting to the mRNA.[1] To achieve the highly efficient gene expression regulating effects of the antisense ODNs, it is required that these molecules pass through the plasma membrane at first. However, charged polar ODNs typically exhibit poor membrane permeabilization because of the hydrophobic membrane barrier, and are therefore inefficiently taken up by cells. Various vehicles have been developed, including cationic lipids, cationic liposomes, and basic peptides.[2] Most of those vehicles, however, facilitate ODN uptake via an endocytic pathway, resulting in the trapping of the ODNs in endocytic compartments and their degradation in lysosomes. There have been attempts at developing a vehicle that enable escape from these compartments.[2] Recently, penetratin,[3] streptolysin O[4], and the cationic derivatives of amphotericin B[5] have been reported as a new class of vehicle that enable passive permeabilization of the plasma membrane without an endocytic process. Although numerous studies have been performed, no perfect vehicle has been developed for clinical use so far. It is vital for antisense therapy to develop a new type of vehicle that operates in a different mechanism. Thus, we attempted to design vehicles in this work.

Peptaibol, which is an antibiotic peptide containing α-aminoisobutyric acid (Aib: U), shows various biological activities related to its channel-forming properties.[6] An 11-residual peptaibol, trichorovin-XIIa (TV-XIIa, Fig. 1), isolated from the fungus *Trichoderma viride*, was found to form pores and channels on planar lipid bilayer membranes.[7] However, the mechanism of the formation remains unclear. Utilizing its membrane-modifying property, we developed a useful vehicle for the delivery of ODNs. Our strategy is as follows: (1) a positively charged ODN linker is covalently conjugated to the

TV-XIIa:
Ac-U-N-I-I-U-P-L-L-U-P-Iol
(Ac: acetyl; U: α-aminoisobutyric acid; Iol; isoleucinol)

Carrier peptide:
Ac-U-N-I-I-U-P-L-L-U-P-I-K-K-K-K-K-K-K-K-K-K-OH

Ac-K_{10}-OH:
Ac- K-K-K-K-K-K-K-K-K-K-OH

Figure 1. Amino acid sequences of the peptides used in the present work.

Keywords: Carrier peptide; Peptaibol; Oligodeoxynucleotide delivery; Antisense.
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C-terminus of TV-XIIa and the conjugate is electrostatically bound to negatively charged ODNs to form complexes; (2) those complexes interact with lipid bilayer membranes and the TV-XIIa-derived carrier peptide forms a pore or channel structure in the membranes; and (3) the entry of ODNs into cells is promoted by the pore or channel formation. We describe herein the design and synthesis of a peptide vehicle and the ODN uptake by cells.

The vehicle was designed to consist of two components: one is a hydrophobic-membrane-interacting, pore- and channel-forming domain derived from the TV-XIIa sequence, and the other is a positively charged ODN binding domain derived from 10-mer of lysine (Fig. 1).[8] Binding between the designed positively charged carrier peptide and the negatively charged ODNs occurs via an electrostatic interaction. It is necessary to form a complex between the vehicle and the ODNs to realize good permeabilization and DNA condensation.[9] The designed carrier peptide was synthesized by the Fmoc-based solid-phase method. Wang resin was used as a solid support. The carrier peptide contains three Aib residues. It is known that the coupling reactivity of Aib with the adjacent α-amino acid is very low in solid-phase synthesis due to the steric hindrance caused by the α,α-dialkyl side chain of Aib. As has been reported previously for the synthesis of Aib-containing peptides, highly reactive amino acid fluorides have been used in the coupling of Aib with the adjacent α-amino acid.[10] We used Fmoc-amino acid fluorides in the coupling reaction of the amino acids at positions 1, 4, 5, 8 and 9. With the exception of the coupling at the above positions, we used 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DOPCDI) as coupling reagents. Deprotection and cleavage of the peptide resin were realized by treatment with trifluoroacetic acid (TFA) containing ethanedithiol and triisopropylsilane. The obtained crude peptide was purified using reversed-phase HPLC and characterized by electro-spray ionization MS.

The ODN used is a 20-mer antisense ODN complementary to positions 1071–1090 of exon 10 of the p53 gene and is fully phosphorothioated in the backbone for protection from degradation by DNase, and has fluorescein (FAM) at the 5′-terminus. The sequence is FAM-5′-CCCTGCTCCCCCCTGGCTCC-3′. As mentioned above, the ability to form complexes with ODN is necessary for good uptake by cells. The peptide–ODN complexation was confirmed by measuring changes in ODN migration in polyacrylamide containing 8 M urea gel.[11] The vehicle associated with ODN does not migrate into the gel during electrophoresis like free ODN does. The binding of the carrier peptide to the ODN was evaluated at various charge ratios between the peptide and the ODN. The experiments were performed by mixing 0.5 μg of ODN with the carrier peptide, followed by incubation of the reaction mixture at 37 °C for 30 min, in which the +(peptide)/−(ODN) charge ratios were increased to 0.5, 1.0, 2.5, 5.0 and 10 (data not shown). The carrier peptide had a slight effect on the migration of the ODN at charge ratios of 0.5 and 1.0, and an increase of the ratio (more than 2.5) led to the complete suppression of the ODN migration. The results indicate that the positively charged lysine side chains of the carrier peptide interacted with the negatively charged phosphate backbone of the ODN, and thus there was no migration of the ODN. The carrier peptide could form a complex with the ODN.

The carrier peptide was tested for its ability to deliver FAM-labelled ODN to mammalian cells, mouse embryonal fibroblast NIH3T3 cells, and human lung carcinoma A549 cells. The carrier peptide and the ODN (0.25 μg: 0.42 μM) were mixed gently in Dulbecco's modified Eagle's medium (DMEM) and incubated at 37 °C for 30 min to allow complex formation. Then, the complex solution was overlaid onto the cells. After incubation at 37 °C for 2 h, the efficiency of the carrier peptide in the delivery of the FAM-labelled ODN and the intracellular localization were determined by confocal laser scanning microscopy (CLSM).[12] Figure 2 shows CLSM images of the complex at various charge ratios. For NIH3T3 cells that were treated with the complex at the charge ratios of 5 (4.2 μM) and 7.5 (6.3 μM), ODN translocation was observed to occur in a dose-dependent manner (Fig. 2C and D). The ODN was essentially accumulated in the cytoplasm and successively passed through the nuclear membrane to reach the nucleus. The ODN was uniformly distributed in the cell interior but not in endocytic compartments. These results indicate that the complex or the ODN might passively permeate through the plasma membrane, diffused in the cytoplasm and eventually reach the nucleus during the incubation for 2 h. The efficiency of the ODN uptake by the cells in the experiment of Figure 2D was estimated by counting more than 200 cells, of which approximately 98% contained the ODN in the cell interiors. Furthermore, the complex uptake by the cells also occurred at 4 °C, which is a condition known to inhibit endocytosis, suggesting that cell permeabilization by the complex does not depend on the endocytic process (Fig. 2F). For the complex at the charge ratio of 10 (8.4 μM), fluorescence was detected in the membranes but not in the cell interior (Fig. 2E). In the control well containing the ODN alone without the carrier peptide, the ODN was taken up by the cells at very low levels, and most of the internalized ODNs were accumulated in peripheral structures, consistent with endosomal localization (Fig. 2A). In addition, one well was treated with Lipofectamine™ (Gibco)/ODN as a positive control (Fig. 2B). Compared with the carrier peptide complex, a high strong fluorescence in the cell interior was observed, but the ODNs were located in endocytic compartments. The lack of nuclear labelling confirmed that the free ODNs were unable to cross the endosomal membranes. In contrast, only a small number of A549 cells showed fluorescence after excitation in the cell interiors with the complexes at the charge ratios of 5 (4.2 μM) and 7.5 (6.3 μM) under the same conditions as those for the NIH3T3 cells, and most of the ODNs were highly accumulated in the membranes (Fig. 2G and H).

The activity of lactate dehydrogenase (LDH), a cytoplasmic enzyme, in the incubation medium was mea-

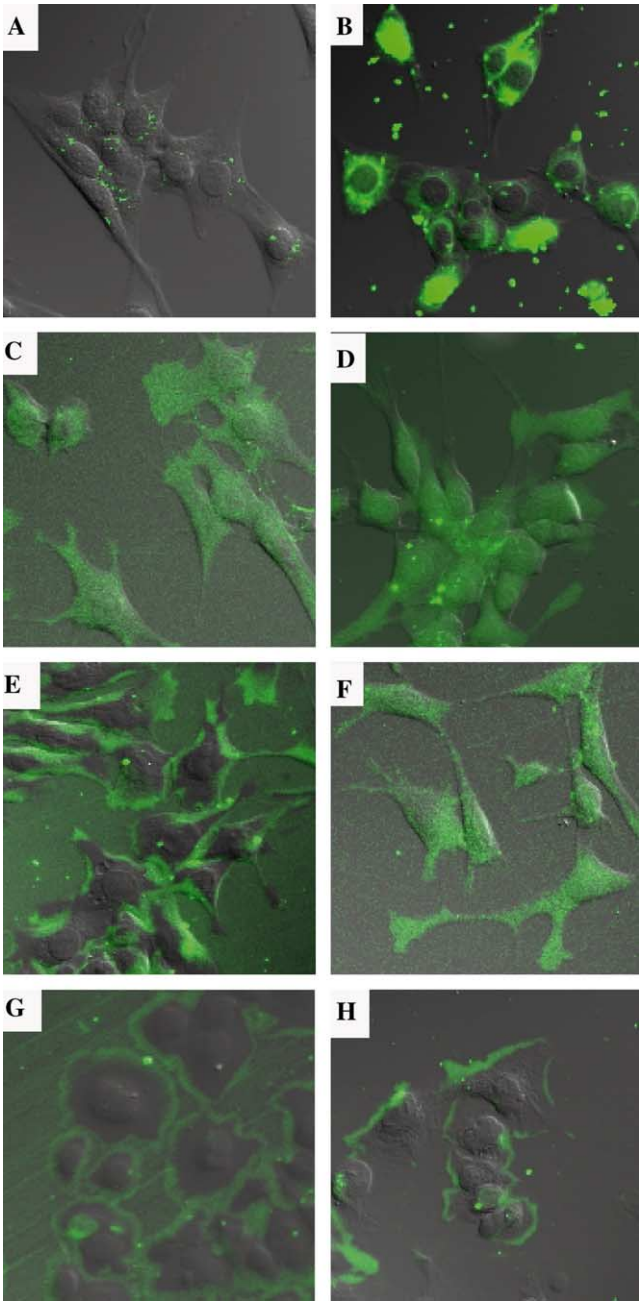

Figure 2. Confocal microscopy images of NIH3T3 (A to F) and A549 (G and H) cells. Fluorescence images and images from transmitted light (differential interference constant) were overlaid for all images. The cells were treated with DNA alone (A), Lipofectamine™/ODN (B) and the carrier peptide/ODN in the charge ratios of 5 (C and G), 7.5 (D and H) and 10 (E) at 37 °C for 2 h, and 5 (F) at 4 °C for 2 h.

sured to determine whether the complex between the carrier peptide and the ODN disrupts the membranes of NIH3T3 cells, thus leading to the leakage of LDH. The exposure of the cells to the complexes at the charge ratios of 5 (4.2 μM) and 7.5 (6.3 μM), which are good conditions for cellular delivery, did not lead to the release of LDH compared to that of control batch. Thus, the complex is not cytotoxic under the conditions examined, and the ODN is translocated into the cytoplasm and the nucleus without causing cell damage.

Polylysine having molecular weight >ca. 10,000 is known to enhance the cellular uptake of ODN.[13] To investigate whether 10-mer lysine, which is used as an ODN linker of the carrier peptide, promotes the cellular uptake of ODN, Ac-K-K-K-K-K-K-K-K-K-K-OH (Ac-K_{10}-OH) was synthesized and assayed for its ability to deliver ODNs into cells. In addition, TV-XIIa, the pore and channel forming domain, was also examined for its ability to enhance the cellular uptake of ODNs. Treatment of NIH3T3 cells with the Ac-K_{10}-OH-ODN complex under the same conditions [charge ratios of 5 (4.2 μM) and 7.5 (6.3 μM)] did not lead to the uptake of ODN by the cells. TV-XIIa (4.2 and 6.3 μM) alone also did not deliver ODN into the cells. These findings suggest that both TV-XIIa and 10-mer-lysine domains in the carrier peptide are structural requirements for promoting the cellular uptake of ODN. At present, it is unclear whether the 10-mer of lysine in the C-terminus of the carrier peptide is purely responsible for the ODN linker, or if the conjugate alone newly acquires the function of cellular delivery.

In conclusion, we have designed a carrier peptide using a pore- and channel-forming peptide. The carrier peptide delivers ODNs into the cytoplasms and nuclei of cells. It may be beneficial for the carrier peptide to deliver ODNs into the nucleus as several lines of evidence have shown that accumulation in the nucleus is important for antisense activity.[14] However, the amount of ODN taken up by the cells is small compared to that of Lipofectamine™. Further structural improvements are necessary. To our knowledge, this is the first report of the design of ODN cellular delivery using peptaibol.

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11. The peptide at the various concentrations and 0.5 μg of the ODN were incubated at 37 °C for 30 min in 50 μL of Dulbecco's modified Eagle's medium (DMEM). Aliquots (10 μL) were collected and analyzed by electrophoresis in 19.5% polyacrylamide gels in the presence of 8 M urea [running conditions: 5.5 mA for 60 min in TBE (Tris–Borate EDTA) running buffer]. Gels were stained with stains-all 4,5,4′,5′-dibenzo-3,3′-diethyl-9-methyl-thiacarbocyanine bromide; 50 μg/mL in 50% formamide solution) for 12 h, and destained in water for 3 h.
12. NIH3T3 and A549 cells were obtained from the Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, and cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640, respectively, supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2. All studies were performed using asynchronous log-phase cultures. Exponentially growing cells were dissociated with trypsin–ethylenediaminetetraacetic acid (EDTA), plated at 50% confluence on glass microscopes slides (Matsunami Glass Ind., Ltd.) and cultured overnight. The culture medium was discarded and the cells were washed three times with DMEM. The cell monolayers were incubated at 37 °C with the complex between the carrier peptide and the ODN for 2 h. Subsequently, the cells were rinsed three times with DMEM at room temperature and fixed with paraformaldehyde [3% (w/v) in phosphate-buffered saline (PBS)] for 10 min at room temperature. For experiments at 4 °C, the protocol was the same except that all incubations were performed at 4 °C until the end of the fixation procedure. Fixed cell monolayers were then washed three times with PBS. The fluorescence distribution was analyzed on a Carl Zeiss LSM510 confocal microscope equipped with a Kr/Ar laser. We also examined with fixed and unfixed cells and the identical intracellular localization of ODN was observed.
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