Carrier PNA for shRNA delivery into cells

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Abstract— A peptide nucleic acid (PNA)-cell-penetrating peptide (CPP) conjugate (carrier PNA) was used as "bridge-builder" to connect a CPP with an shRNA. The carrier PNA successfully formed a hybrid with an shRNA bearing complementary dangling bases and the shRNA was introduced into cells by the carrier PNA, and RNAi was induced by the shRNA.

Short double-stranded RNAs, termed small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs), decompose a target mRNA sequence-specifically on the basis of RNA interference (RNAi) and downregulate the specific gene expression in cells.[1] RNAi is commonly used as a tool to analyze a genetic function in biochemical and medical fields. These RNAs are attractive therapeutic candidates for interferring with the expression of undesirable genes. Since siRNA and shRNA cannot introduce themselves into cells, various delivery systems for carrying these RNAs have been developed.[3] Of these delivery systems, some delivery system using cell-penetrating peptides (CPPs) as the carriers for RNAs have been reported[4] and several studies have shown that siRNAs connected with CPPs (siRNA-CPPs) entered cells.[5] The advantage of this method is that an excess amount of RNA carrier reagents is not necessary due to the direct connection between the siRNAs and the CPP. However, this method needs time-consuming synthesis of the siRNA-CPP for every siRNA. A simpler way to connect the CPP and every RNA is needed.

In this report, we describe a new method to connect an siRNA or shRNA with a CPP more simply and rapidly. To achieve this purpose, we utilized a peptide nucleic acid (PNA).[6] The PNA is a peptide-based nucleic acid surrogate consisting of nucleobases in the side chain, and the PNA can form a more stable hybrid with a complementary nucleic acid than nucleic acids themselves because of the absence of electrostatic repulsion between PNAs and nucleic acids. First, we synthesized a PNA conjugated to a CPP (carrier PNA). Synthesis of a PNA conjugated to a CPP was much easier than synthesis of an siRNA-CPP because both the PNA and the CPP can be connected by amide bonds from solid-phase peptide synthesis (SPPS). Next, we synthesized an shRNA bearing complementary dangling bases to the PNA moiety in the carrier PNA. When the carrier PNA is mixed with the shRNA, the shRNA easily connects to the CPP, resulting in the formation of a hybrid between the dangling bases and the PNA moiety. This method also has the advantage that the carrier PNA can be utilized as a carrier of any shRNA and siRNA bearing complementary dangling bases. In general, a PNA conjugated to a CPP is used as an antisense agent,[7] whereas we used the carrier PNA as a "bridge-builder" to connect a CPP with an shRNA in this work. The carrier PNA was synthesized by Fmoc-based SPPS.

The product was identified by MALDI-TOF Mass (calcd. $[M+H]^+$ = 3909.87, obsd. $[M+H]^+$ = 3909.92, see supplementary information Fig. S1). The chemical structure of the carrier PNA is shown in Fig. 1. The sequence of PNA is N'-TGGTGCGAA-C' and the CPP is octaarginine (R8). An R8 efficiently translocates through the cell membranes and works as a carrier.[8] An R8 (H-R8-NH_2, MALDI-TOF Mass; calcd. $[M+H]^+$ = 1266.84, obsd. $[M+H]^+$ = 1266.96) and a carrier PNA that consists of a different sequence of PNA (carrier misPNA; H-PNA(GCAGTGAGT)-ehyleneglycol

spacer-R8-NH_2, MALDI-TOF Mass; calcd. $[M+H]^+$ = 3909.87, obsd. $[M+H]^+$ = 3909.17) were also synthesized as references. The structure of an shRNA targeted to the firefly luciferase gene (anti-Luc shRNA) is also shown in Fig. 1. The anti-Luc shRNA was obtained by transcription using T7 RNA polymerase.[9] Dangling bases of the 5' end in the anti-Luc shRNA are complementary with the PNA moiety in the carrier PNA.

We preliminary investigated whether the carrier PNA formed hybrids with nucleic acids. The UV melting curve of an equimolar mixture of the carrier PNA and a complementary DNA is shown in Fig 2.[10] In the case of detection at 260 nm derived from nucleobases, it was shown that the carrier PNA formed a hybrid with a complementary DNA. The melting temperature (T_m) was 70.6 ˚C. A UV melting curve of an equimolar mixture of carrier PNA without an R8 (H-PNA(TGGTGCGAA)-NH_2) and the complementary DNA was also measured. The T_m value was 68.6 ˚C. These results indicate that the conjugation of a cationic R8 to a PNA does not noticeably affect hybridization of the PNA with a complementary DNA. A similar result was also reported in a paper on duplex stability of DNAs conjugated to a cationic oligomer and a complementary DNA.[11] Although a UV melting curve for an equimolar mixture of the carrier PNA and a mismatched DNA was like a sigmoid curve (detection at 260 nm), absorbance at 350 nm increased at low temperatures, suggesting that aggregation of the carrier PNAs and the mismatched DNAs was promoted due to electrostatic interactions between CPPs and DNAs instead of the formation of a hybrid. Such a rise of turbidity was not observed between the carrier PNA and the complementary DNA. These results suggest that hybridization between the PNA moiety in the carrier PNA and the complementary DNA preferentially proceeded compared to the nonspecific interaction between cationic CPPs and anionic DNAs. These results also suggest that the carrier PNA will sequence-specifically transport nucleic acids into cells.

Cellular internalization of the hybrid between the carrier PNA and a longer DNA was examined by confocal laser-scanning microscopy.[12] A fluorescence image of Chinese hamster ovary (CHO) cells obtain after equilibration with hybrids of the carrier PNA with an FAM-labeled DNA bearing complementary bases to the PNA moiety is shown in Fig. 3a. The intracellular location of the hybrid was mostly confined in small vesicular compartments. This result indicates that the hybrid is successfully introduced into the cells by the endosomal pathway. On the other hand, the FAM-labeled DNA was not internalized with an R8 by the cells under same conditions (Fig. 3b). In addition, the FAM-labeled DNA alone was not internalized by the cells. These results suggest that the PNA moiety has an important role in the introduction of specific nucleic acids into cells. In other words, these results indicate that the nucleic acids were introduced through the carrier PNA into cells when the PNA moiety formed a hybrid with the nucleic acid.

We investigated the RNAi effect of an shRNA with the carrier PNA in cells.[13] The carrier PNA and then an anti-Luc shRNA were added to the medium supplemented with fetal bovine serum (FBS) on CHO cells expressing firefly luciferase (CHO-AA8-Luc cells), and the cells were incubated. The final concentrations of a mixture of the carrier PNA and the anti-Luc shRNA were 250 nM and 500 nM, respectively. Fig. 4a shows luciferase expression in the cells. As negative control experiments, we also measured luciferase activities of cells incubated with the anti-Luc shRNA alone and cells incubated with a mixture of the anti-Luc shRNA and a carrier PNA containing a mismatched PNA sequence (carrier misPNA). Compared to the result of the negative control experiments, the anti-Luc shRNA with the carrier PNA suppressed luciferase expression to 22% in the case of 250 nM of the hybrid and to 38% in the case of 500 nM of the hybrid.[14] Fig. 4b shows that the RNAi effect was observed 18-24 h after incubation with the carrier PNA/shRNA hybrid. These results suggest that the carrier PNA introduced the shRNA into cells. The mixture of the carrier misPNA and the anti-Luc shRNA did not suppress luciferase expression, indicating that the shRNA was successfully introduced into cells by the carrier PNA via formation of a sequence-specific PNA/RNA hybrid.

In summary, we have developed the new method to introduce an shRNA (or siRNA) into cells. The carrier PNA successfully formed a hybrid with an shRNA bearing complementary dangling bases and the shRNA was introduced into cells by the carrier PNA, and then RNAi was induced by the shRNA. When shRNAs and siRNAs bearing complementary dangling bases are prepared, any kinds of RNAs will be introduced by the carrier PNA into cells. Escape of the carrier PNA/shRNA hybrid from the endosome is currently under investigation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: xx.xxxx/j.bmcl.xxxx.xx.xxx.

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9. To generate a DNA template for transcription, primer extension was performed using two primers, which were designed to complement each other at each 3' region: 5'-CCGGGTAATACGACTCACTATAGTTCGCACCAGG TTGTGTCTGGTTGTGTAAAGCTTCC -3' and 5'-AAGATTATGTCCGGTTATGTAAAGTGACAGGAAG CTTTACACAACCAG -3'. In the DNA template, the promoter sequence for T7 RNA polymerase is inserted directly upstream of the shRNA sequence. The synthetic shRNA having the sequence shown in Fig. 1 was prepared by T7 transcription at 37 ˚C for 5 h in a reaction mixture containing 40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM spermine, 0.01% Triton X-100, 50 μg/mL BSA, 10 mM GMP, 2 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 20 μg/mL T7 RNA polymerase and 50 nM template double-stranded DNA. The product was purified by 10% denaturing polyacrylamide gel electrophoresis (PAGE).

10. The UV melting curves were recorded with cooling of the solution at 0.5 ˚C/0.5 min. Each concentration of the carrier PNA and DNAs was 5 μM. UV experiments were conducted in an aqueous buffer (100 mM NaCl, 10 mM NaH_2PO_4 and 0.1 mM EDTA, pH 7.0). The observed absorbance has been normalized at 80 ˚C.

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12. CHO cells were cultured in Dulbecco's modified Eagle's medium. The medium was supplemented with penicillin/streptomycin (10 μg/mL) and 10% FBS. The cells were incubated at 37 ˚C under 5% CO_2 to give ~70% confluence. Then subculture was performed on 35 mm glass-based dishes that were coated with poly(L-lysine). The cells were incubated at 37 ˚C under 5% CO_2 until 40~60% confluence. Before cellular uptake, the cells were incubated at 37 ˚C for 2 h on a fresh medium containing the carrier PNA/ FAM-labeled DNA hybrid. The final concentration of the hybrid in the medium was 10 μM. The cells were then washed three times with PBS and examined under a confocal laser-scanning microscope without fixation.

13. CHO-AA8-Luc Tet-Off cells (Clontech) were grown at 37 ˚C under 5% CO_2 in Ham's F-12 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Forty-eight hours before transfection at 80% confluence, the cells were trypsinized, diluted with fresh medium (8×10^4 cells/mL), and transferred to 96-well plates (100 μL per well). Transfection of the anti-Luc shRNA in CHO-AA8-Luc cells was carried out by separately adding the carrier PNA (250-500 nM) and then the anti-Luc shRNA (500 nM) into F-12 medium containing 10% FBS. At 2-24 h after the addition of the carrier PNA and the anti-Luc shRNA, cells were lysed by Passive Lysis Buffer (Promega) and divided for subsequent experiments. Firefly luciferase expression

in CHO-AA8-Luc cells was measured by FLUOstar OPTIMA (BMG Labtech, Germany) with the addition of a Luciferase Assay Reagent II (Promega). Firefly luciferase expression efficiency in each well was normalized by total protein amount, which was quantified from the remaining lysate by the a Protein Assay Kit (BIO-RAD, CA, USA).

14. We also investigated the RNAi effect of the anti-Luc shRNA with a transfection reagent (TransIT-TKO®) under same conditions. In the case of 500 nM of the mixture, the anti-Luc shRNA with TransIT-TKO® suppressed luciferase expression to 94%.

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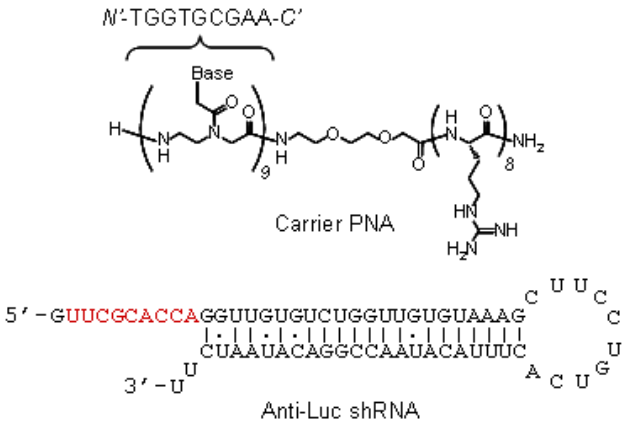

Figure 1. Structures of the carrier PNA and the anti-Luc shRNA. A PNA is conjugated to a CPP via an ethyleneglycol linker as a spacer. Nine dangling bases of the 5' end of anti-Luc shRNA are shown in red. The bases are complementary with the PNA moiety in the carrier PNA.

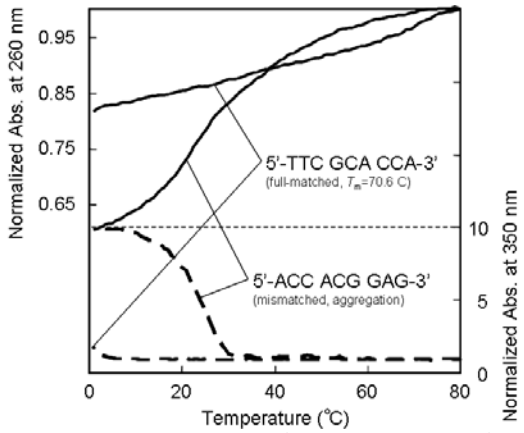

Figure 2. UV melting curves of equimolar mixtures of the carrier PNA and DNAs. Solid lines are curves detected at 260 nm, and dotted lines are curves detected at 350 nm. The base sequence of the complementary DNA is 5'-TTCGCACCA-3', and the base sequence of the mismatched DNA is 5'-ACCACGGAG-3'.

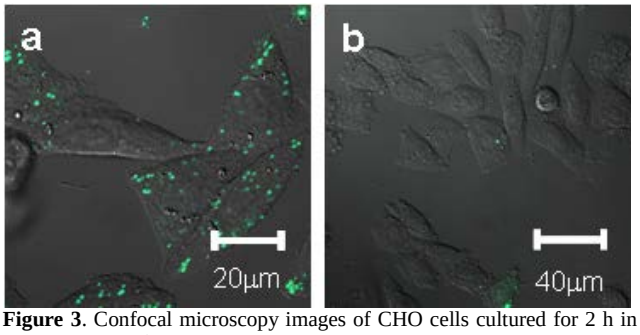

Figure 3. Confocal microscopy images of CHO cells cultured for 2 h in the presence of 27-mer FAM-labeled DNA bearing complementary dangling bases (5'-FAM-CTT CTC AGT TAG GGT TAG TTC GCA CCA-3': complementary sequence underlined) with the carrier PNA (a) and with the R8 (b).[12]

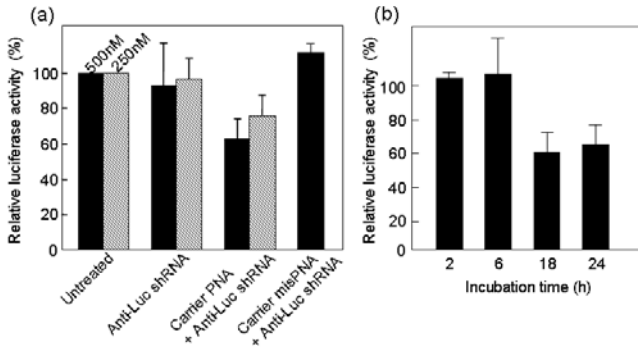

Figure 4. (a) RNAi-mediated silencing of firefly luciferase gene in CHO-AA8-Luc cells by an anti-Luc shRNA with the carrier PNA. The cells were incubated with the shRNA and the carrier PNA for 24 h in a medium supplemented with 10% FBS. (b) Time dependence of RNAi by 500 nM carrier PNA/shRNA hybrid.