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A leucine zipper-based peptide hybrid delivers functional Nanog protein inside the cell nucleus

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ABSTRACT

We synthesized a pair of compounds containing leucine zipper peptides to deliver protein cargo into cells. One is a cell-penetrating peptide (CPP) with Lz(E), a leucine zipper peptide containing negatively charged amino acids, and the other is a Nanog protein with Lz(K), a leucine zipper peptide containing positively charged amino acids. When cells were treated with these equimolar mixtures, Nanog-Lz(K) hybridized with Lz(E)-CPP was successfully delivered into the cells. Furthermore, Nanog-Lz(K) exerted its proper function after nuclear transport.

It is important to develop techniques for delivering proteins or peptides with specific functions into cells because the techniques are expected to be used for protein- or peptide-based drugs against numerous molecular targets within the cells that cause certain diseases. Most current protein- or peptide-based drugs including therapeutic antibodies are subject to limitations in that the targets are molecules on the cell surface, e.g. receptor proteins. Among the techniques, cell-penetrating peptides (CPPs) are widely used for protein or peptide delivery into cells. This delivery method is beneficial since, by genetic engineering or solid-phase peptide synthesis, the CPPs can easily be conjugated to proteins or peptides via amide linkage.[1,2] However, direct conjugation of the carrier CPPs with the cargo peptides or proteins may cause problems after they are delivered into the cells because CPPs consist of several positively charged amino acids and consequently interact nonspecifically with negatively charged nucleic acids and membrane proteins inside the cells, leading to interference with the biological function of cargo adjacent to the CPPs.[3] Therefore, the conjugate needs to connect the carrier with the cargo until delivery into the cell and separate the cargo from the carrier after delivery inside the cell.

So far, we have developed a novel method of CPP-based intracellular delivery to mitigate this problem. In our method, we used leucine zipper (Lz) peptides containing glutamic acids (Lz(E)) or lysines (Lz(K)), respectively.[4] These two types of Lz peptides form a heterodimeric hybrid with a molecular ratio of 1:1 and the Lz region in the hybrid works as molecular glue between a functional peptide cargo conjugated to Lz(K) and a CPP conjugated with Lz(E).[5] Such molecular glue with a coiled-coil motif is a reliable method utilized by numerous researchers.[6–13] Heterodimers spontaneously dissociate inside the cells since the interaction of Lz(K) with Lz(E) is dependent on the hybrid concentration. Of note, because the cargo is not covalently linked to the CPP in this system, it is free from the negative effect of CPP after dissociation within the cells. Using this method, we showed that an autophagy-inducing peptide was successfully delivered into cells and the transduced peptide exerted its proper function in the cytoplasm without any detectable side effects while the cells treated with the same peptide directly conjugated with the CPP resulted in undesired severe cell death, indicating the usefulness of the Lz-based hybrid method for functional peptide delivery.[4]

However, it remains unclear whether proteins with a larger molecular weight than peptides can also be delivered into cells by the same method. The establishment of protein delivery by this method is useful not only for intracellular chemotherapy, but also for artificial modification of cells, leading to the generation of the desired types of cells such as induced pluripotent stem (iPS) cells without any risk of chromosome damage observed when DNA materials are used in such trials.[14] Furthermore, it also remains unknown whether a nuclear protein delivered by the Lz-based hybrid method enters the nucleus and properly executes its function. In the present study, we investigated

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Fam-Lz(K): Fam-EYQALKKKVAQLKAKNQALKKKVAQLKHK-bA

CPP-Tmr-Lz(E): RRRRRRRRRRR-K(Tmr)-
EYQALEKEVAQLEAENQALEKEVAQLEHE-bA

CPP-Tmr-Lz(K): RRRRRRRRRRR-K(Tmr)-
EYQALKKKVAQLKAKNQALKKKVAQLKHK-bA

Tmr-Lz(E): Tmr-EYQALEKEVAQLEAENQALEKEVAQLEHE-bA

Nanog-Lz(K): Nanog protein—EYQALKKKVAQLKAKNQALKKKVAQLKHK

Lz(E)-CPP: EYQALEKEVAQLEAENQALEKEVAQLEHE-
RRRRRRRRRRR-bA

Lz(E): EYQALEKEVAQLEAENQALEKEVAQLEHE-bA

Fig. 1. Sequences of four fluorescence-modified Lz(K) and Lz(E) (Fam-Lz(K), CPP-Tmr-Lz(E), CPP-Tmr-Lz(K) and Tmr-Lz(E)), Lz(K) conjugated to Nanog protein (Nanog-Lz(K)), Lz(E) conjugated to undeca-arginine (Lz(E)-CPP) and Lz (E). The red letters indicate the charged amino acids critical for forming a hybrid between Lz(K) and Lz(E). bA represents b-alanine. Lz(E) is a negative control for Lz(E)-CPP. Chemical structures of Fam, Tmr and K(Tmr) are shown in Fig. S1.

whether our Lz-based hybrid method works successfully for the delivery of the functional nuclear protein.

We initially assessed the capability of the Lz-based hybrid system as a method for intracellular delivery by using simple Lz peptides labelled with fluorescence dyes. Fam-Lz(K) and Tmr-Lz(E) were prepared as described in the previous report and CPP-Tmr-Lz(E) and CPP-Tmr-Lz(K) were newly prepared by conventional solid-phase peptide synthesis (Fig. 1).[4] The CPP-Tmr-Lz(E) peptide is an Lz(E) peptide conjugated to undeca-arginine as a CPP and Lys(Tmr) at the N-terminus. We also prepared CPP-Tmr-Lz(K) in which Lz(E) in CPP-Tmr-Lz(E) was replaced with Lz(K). The hybrids were formed by mixing the proper combination of these peptides at a molecular ratio of 1:1. Then, the hybrids were incubated to U-251 cells for 4 h before washing and the cells were observed under a confocal microscope (Fig. 2). The cells treated with the hybrid consisting of Fam-Lz(K) and CPP-Tmr-Lz(E) showed the cytoplasmic localization pattern of both the Fam and Tmr signals, indicating that Lz(K) and Lz(E) were internalized into cells (Fig. 2a). On the other hand, in the cells treated with the mixture of Fam-Lz(K) and CPP-Tmr-Lz(K), only the Tmr signal was observed in the cytoplasm and the Fam signal was almost undetectable (Fig. 2b). These results suggested that Fam-Lz(K) is delivered into the cells by hybridization with CPP-Tmr-Lz(E) and the hybridization was via the interaction of Lz(K) and Lz(E). In the cells treated with the hybrid consisting of Fam-Lz(K) and Tmr-Lz(E), the Fam signal was not significantly observed while the Tmr signal was clearly detected. This Tmr signal, however, seemed to concentrate on the cell surface but not in the cytoplasm (Fig. 2c). It is unclear why only the Tmr-Lz(E) peptide but not the Fam-Lz(K) adhered to the cell surface in this experimental setting. It might be possible that the hybrid of Tmr-Lz(E) with Fam-Lz(K) stuck to the cell surface via Tmr moiety as cholesterol moiety, and only Fam-Lz(K) was dissociated after the washing process, whereas Tmr-Lz(E) stayed on the cell surface.[9–12] The accumulation of Tmr-Lz(E) peptide on the cell surface indicated that the conjugation of CPP to Lz peptide is absolutely required for internalization of the hybrid of Fam-Lz(K) with Tmr-Lz(E) into cells. Based on these results, we used Lz(E)-CPP as a carrier for delivery of a recombinant nuclear protein into cells as follows. Considering the possible steric hindrance between CPP and a nuclear protein, the position of the CPP relative to Lz(E) has been altered from the N-terminus to the C-terminus.

As a model cargo nuclear protein, we decided to use Nanog protein. Nanog is one of the transcription factors present in the nuclei and is involved in the pluripotency of undifferentiated embryonic stem cells.[15,16] To prepare recombinant Nanog protein conjugated to Lz(K) at the C-terminus (Figure 1), the PCR product corresponding to the Nanog-Lz(K) DNA was amplified and cloned into the pET28a(+) vector. After transformation of DH5α with the resultant plasmid, the expression of Nanog-Lz(K) protein was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG). Because this protein also possesses His-tag derived from the pET vector, the Nanog-Lz(K) protein was highly purified with

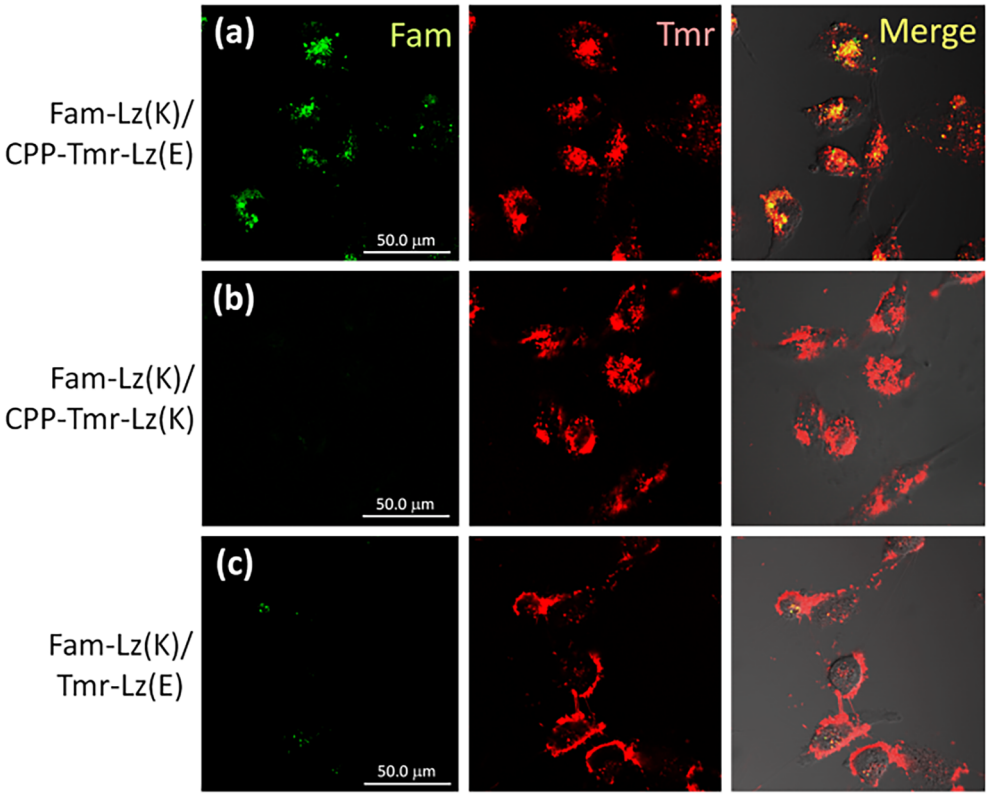

Fig. 2. Confocal microscopy images of U-251 MG cells treated with Lz-based hybrids. The cells were treated with 10 μM of the respective hybrids, (a) Fam-Lz(K) and CPP-Tmr-Lz(E), (b) Fam-Lz(K) and CPP-Tmr-Lz (K), and (c) Fam-Lz(K) and Tmr-Lz(E) for 4 h. Fam and Tmr fluorescence signals were separately detected as red and green signals, respectively. The merged images are also included in the right panel (yellow signal).

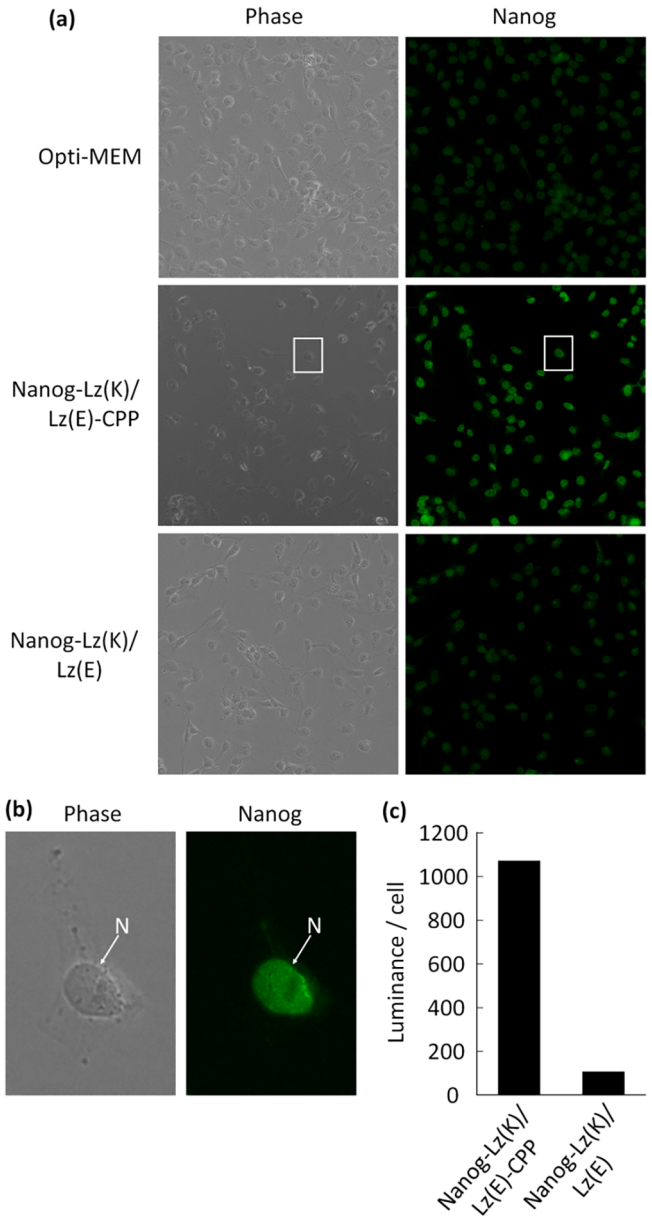

Fig. 3. (a) Confocal microscopy images of HeLa cells treated with Opti-MEM (medium control), the hybrid consisting of Nanog-Lz(K) and Lz(E)-CPP (each final concentration was 5 μM), or the hybrid of Nanog-Lz(K) and Lz(E). The cells were incubated for 2 h at 37°C before cell fixation. The cells were detected by immunofluorescence microscopy. (b) Enlarged views of the white squares in (a). Nuclear localization of the Nanog protein is evident. (c) The cell number and fluorescence intensity of cells detected in (a) were measured and the luminance per cell was calculated (y-axis, luminance/cell). The mean values from n = 10 were calculated for each sample and the background signal was subtracted, which was the mean signal of the Opti-MEM sample.

a nickel-chelating affinity column. The purified protein was assessed by Coomassie Blue staining following SDS-PAGE (Fig. S4). In addition to Nanog-Lz(K) and Lz(E)-CPP, we also synthesized Lz(E) peptide without CPP as a negative control (Fig. 1). These peptides were prepared by conventional solid-phase peptide synthesis and identified with MALDI-TOF mass spectrometry and RP-HPLC (See Supporting Information).

To investigate intracellular delivery of the recombinant Nanog protein by using the Lz-based hybrid method, we incubated HeLa cells with the 1:1 mixture of purified Nanog-Lz(K) and Lz(E)-CPP or the mixture of Nanog-Lz(K) and Lz(E) for 2 h. We used the 1:1 ratio since Lz (K) binds to Lz(E) at molecular ratio of 1:1 according to our previous titration assay.[4] After fixation and permeabilization, the cells were treated with anti-His tag specific antibody followed by staining with fluorescent-labelled secondary antibody (Fig. 3a-c). We did not use an anti-Nanog antibody because of the possible expression of Nanog in the HeLa cells. The results showed that Nanog-Lz(K) protein was clearly detected inside the cells treated with Nanog-Lz(K)/Lz(E)-CPP, indicating proper internalization of Nanog-Lz(K) into the cells (Fig. 3a). Furthermore, the enlarged image shows the nuclear staining pattern of Nanog-Lz(K) protein (Fig. 3b). On the other hand, in the case of the hybrid consisting of Nanog-Lz(K) and Lz(E), the level of Nanog-Lz(K) signal was only a background level since the signal was similar to that of the medium control sample (Fig. 3a, Opti-MEM). These results indicate that the transduction of Nanog protein into cells is dependent on the CPP in Lz(E)-CPP and Nanog protein delivered by the Lz-based hybrid method retains its intrinsic properties in terms of nuclear localization.

To evaluate whether the Nanog protein delivered into the nucleus retains its bioactivity as a transcriptional regulator, we investigated the mRNA expression of Rnd3 in the presence or absence of Nanog since Rnd3 is known to be a target of Nanog and is suppressed at the transcription level in HeLa cells.[17] The total RNAs were obtained from the cells treated with the Lz-based hybrids and were reverse transcribed for the following quantitative RT-PCR (qPCR) (Fig. 4). The qPCR was performed with the standard SYBR Green I protocol using Rnd3 specific oligos on a Step One Plus System (Applied Biosystems). Treatment of cells with the hybrid consisting of Nanog-Lz(K) and Lz(E) did not reduce the Rnd3 mRNA expression compared to that in the Opti-MEM medium control. On the other hand, the Rnd3 mRNA level in the cells treated with the Nanog-Lz(K) and Lz(E)-CPP hybrid was decreased. These results suggested that Nanog protein delivered into the nuclei by our Lz-based hybrid system obviously retains its biological function.

In summary, we utilized leucine zipper motifs Lz(E) and Lz(K), which form the hybrid at a molecular ratio of 1:1 as a delivery tool for Nanog protein, a larger molecule than peptides. The cargo protein was noncovalently connected with the CPP in the hybrid. Nanog protein was successfully delivered into the cells by the hybrid, and was

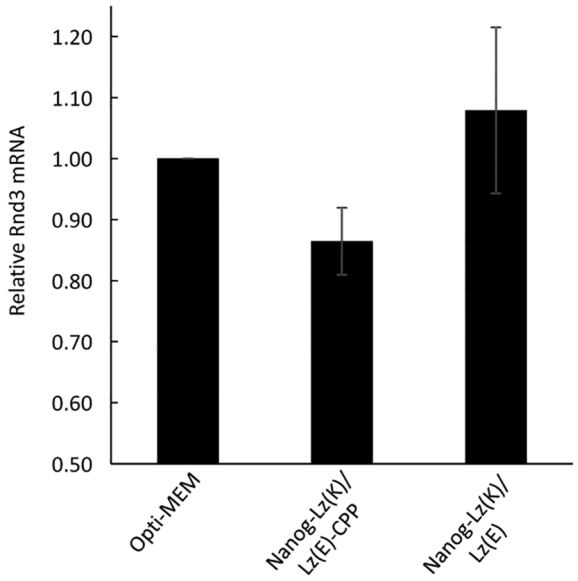

Fig. 4. Rnd3 mRNA expression level in the presence of Nanog. HeLa cells were incubated with Opti-MEM (control), the hybrid of Nanog-Lz(K)/Lz(E)-CPP (each final concentration was 10 μM), or the hybrid of Nanog-Lz(K)/Lz(E) at 37 °C for 2 h. The cells were further incubated for 2 days and then harvested to extract total RNAs. RNAs were reverse transcribed and subjected to qPCR. The qPCR experiments were done in triplicate and at least three independent experiments were performed. Representative data with the means and SE of triplicates is shown.

subsequently transported into the nucleus. Furthermore, the Nanog protein in the nucleus was still bioactive. Treatment of cells with the Nanog-Lz(K)/LZ(E)-CPP complex did not significantly alter the cell morphology. Further, these cells treated with the complex retained intact cellular response in terms of Rnd3 expression at 2 days after the complex treatment. These results indicate that the Nanog-Lz(K)/LZ(E)-CPP treatment is hardly cytotoxic. In this study, we used Nanog as a model nuclear protein and HeLa cell as a model cell for evaluation of the Lz-based protein delivery system. It is important to test in the next step whether this strategy can adapt to other nuclear proteins and cells. Especially, the Lz-based hybrid strategy for protein delivery into cells will be applied to other proteins including master transcriptional regulators to generate specific types of cells and therapeutic antibodies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.02.004.

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