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Identification of a Novel Cell-Penetrating Peptide from Human Phosphatidate Phosphatase LPIN3

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Biomolecules such as proteins, DNA, and RNA are macromolecules and can not cross the cell membrane. However, cell-penetrating peptide (CPP) has been shown to deliver therapeutic biomolecules successfully into cells. The various and widely used CPPs including TAT, VP22, and Antp are mostly non-human originated CPPs, and are limited by their potential toxicity and immunogenicity. We report here on a newly identified novel cell-penetrating sequence (LPIN; RRKRRRRRK) from the nuclear localization sequence (NLS) of human nuclear phosphatase, LPIN3. LPIN-EGFP recombinant protein was concentration- and time-dependently delivered into cells and localized to the nucleus as well as the cytoplasm. It penetrated the cell membrane by lipid raft-mediated endocytosis by binding to heparan sulfate proteoglycan. LPIN-EGFP was successfully delivered into primary mouse splenocytes *in vitro* and it could be delivered into various tissues including liver, kidney, and intestine in mice after intra-peritoneal injection. This research suggests that LPIN-CPP could be used in a drug delivery system to deliver therapeutic biomolecules including peptides, proteins, DNA, and RNA and without the limitations of non-human originated CPPs such as TAT-CPP.

INTRODUCTION

Macromolecules such as proteins, DNA, and RNA, are limited by their inability to penetrate the selectively permeable cell membrane. Various drug delivery systems have been proposed to deliver therapeutic bio-molecules into cells efficiently *in vivo* including liposomes, nano-particles, cell penetrating peptides, and others (Ogris and Wagner, 2002; Torchilin, 2005).

Cell penetrating peptides (CPPs) or protein transduction domains (PTDs) are small cationic peptides which have been suggested for use as a delivery method to introduce therapeutic bio-molecules into cells (Snyder and Dowdy, 2004). They are mostly derived from non-human-originated molecules such as viruses, drosophila, etc. HIV-derived TAT (Frankel and Pabo, 1988; Schwarze et al., 1999), herpes simplex virus-derived VP22 (Elliott and O'Hare, 1997; Morris et al., 2001), and drosophila-derived Antp (Derossi et al., 1994) were initially identified and widely used as cell-penetrating sequences for the purpose of research and medicine. The major transduction mechanisms of these peptides were suggested to be endocytosis, direct penetration, and micelle formation (Richard et al., 2003; van den Berg and Dowdy, 2011). Although there has been remarkable transduction efficiency and good therapeutic effects noted for cargo molecules *in vitro*, *in vivo* application has been much more limited because of toxicity, immunogenicity, and low delivery efficiency. Recently, human-derived CPPs such as Hph-1 (Choi et al., 2006), Sim-2 (Choi et al., 2012), lactoferrin (Duchardt et al., 2009), and vectocell (De Coupade et al., 2005) were identified, however their delivery efficiencies are typically lower than that of TAT and still, *in vivo* delivery efficiency was not sufficient. Therefore, the development of novel human-originated CPPs with a higher transduction efficiency is still required.

LPIN3 is a human phosphatidate phosphatase, which has phosphatidate phosphatase type-1 (PAP1) activity and has a key role in glycerolipid metabolism (Donkor et al., 2007; Reue and Brindley, 2008). In the present study, we have identified a cell-penetrating amino acid sequence from LPIN3 and proved its delivery efficiency *in vitro* and *in vivo*. Bioinformatic screening of homology searching with TAT-CPP generated a nuclear localization sequence (NLS) of LPIN3 (LPIN) as a candidate CPP. LPIN conjugated recombinant enhanced green fluorescence protein (EGFP) was purified for the analysis of its intracellular transduction efficiency *in vitro* and *in vivo*. Its transduction efficiency and intracellular localization was analyzed in Jurkat T cells and HeLa cells, and its delivery mechanism was suggested as lipid raft-mediated endocytosis after binding to heparan sulfate proteoglycan on the cell surface. In addition, intra-peritoneal injection of LPIN-EGFP showed significant *in vivo* penetration activity into various tissues including kidney, liver, and intestine, suggesting that LPIN-CPP is a novel human-originated cell-penetrating sequence that could be used for therapeutic purposes with various cargo molecules.

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MATERIALS AND METHODS

Purification of recombinant CPP-EGFP proteins

After CPP-EGFP DNAs were cloned into pRSET-B vector, *E. coli* BL21 (DE3) star pLysS cells were transformed with the constructed plasmids. Colonies were inoculated into 50 ml of Luria-Bertani media broth (LB broth) containing ampicillin (50 μg/ml) and grown for 12 h at 37°C. The 50-ml cultures were transferred into 500 ml of fresh LB medium and grown at 37°C for 1-2 h until the optical density (O.D.) reached between 0.4-0.6 at 600 nm. Bacterial protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 20°C for overnight. Bacterial cells were harvested by centrifugation at 6,000 rpm for 20 min at 4°C. 6-His-tagged target proteins were purified through Ni-NTA affinity chromatography (Qiagen) and desalted using a PD-10 column (Amersham).

Cell culture

The Jurkat (E6.1) cell line was purchased from ATCC. Cells were maintained in RPMI media (Welgene) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics. The HeLa cell line was cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics. All cells were cultured at 37°C in a 5% CO_2 incubator.

Analysis of intracellular delivery efficiency of LPIN-EGFP

The 5.0×10^5 Jurkat cells per well were cultured in 24-well plates. CPP-EGFP proteins were added to each well and incubated for an additional period of time, depending on the particular experiment. Following incubation, the cells were harvested and washed three times with PBS. Intracellular fluorescence was analyzed by flowcytometry (FACS Canto, BD Bioscience). Data were analyzed using Flowjo software ver. 8.2 (Tree star, Inc.).

Visualization of intracellular localization of LPIN-EGFP

The 1.0×10^6 HeLa cells per well were seeded in 6-well plates in DMEM media for overnight. HeLa cells were treated with LPIN-EGFP (20 μM) for 2 h and then the cells were washed five times with PBS. These cells were fixed with 4% paraformaldehyde and stained with Hoechst 33342 (Invitrogen) for nucleus staining. After washing three times with PBS, cells were mounted on glass slides and the images were analyzed by fluorescence or confocal microscopy.

Visualization of tissue delivery of LPIN-EGFP in mice

Six-week-old C57BL/6 mice were purchased from DHbiolink (DBL, Korea). These mice were intraperitoneally injected with 5 mg of LPIN-EGFP or control protein. One to 2 h after the injection, the mice were sacrificed and the tissues were harvested. The tissues were washed with PBS and fixed with 4% paraformaldehyde. Then, frozen blocks were produced with O.C.T. compound (Wako). All blocks were cut into 5-μm thick slices and observed with fluorescence microscopy.

Statistics

Data were analyzed by two-tailed Student's *t*-test. P-values of less than 0.05 were considered statistically significant.

RESULTS

Generation of LPIN-EGFP

Nine amino acids of the NLS region on the LPIN3 protein (141-149, RRKRRRRRK, LPIN) were selected to be the candidate sequence for a cell-penetrating sequence because of their similarity with TAT-CPP, which is rich with cationic amino acids (Fig. 1A). LPIN-EGFP and control DNAs were constructed into pRSET-B plasmid (Fig. 1B) and recombinant proteins were purified in the BL-21 (DE3) strain of *E. coli* (Fig. 1C). The widely used cationic cell-penetrating sequence of TAT-CPP and the human-derived cationic sequence of Hph-1-CPP were used as control proteins for comparison.

Intracellular delivery of LPIN-EGFP into Jurkat T cells

To determine the protein delivery efficiency of LPIN-CPP, 1-5 μM LPIN-EGFP was added to Jurkat T cells for 2 h and then the intracellular fluorescence was analyzed by flowcytometry (Fig. 2A). LPIN-EGFP showed concentration-dependent intra-

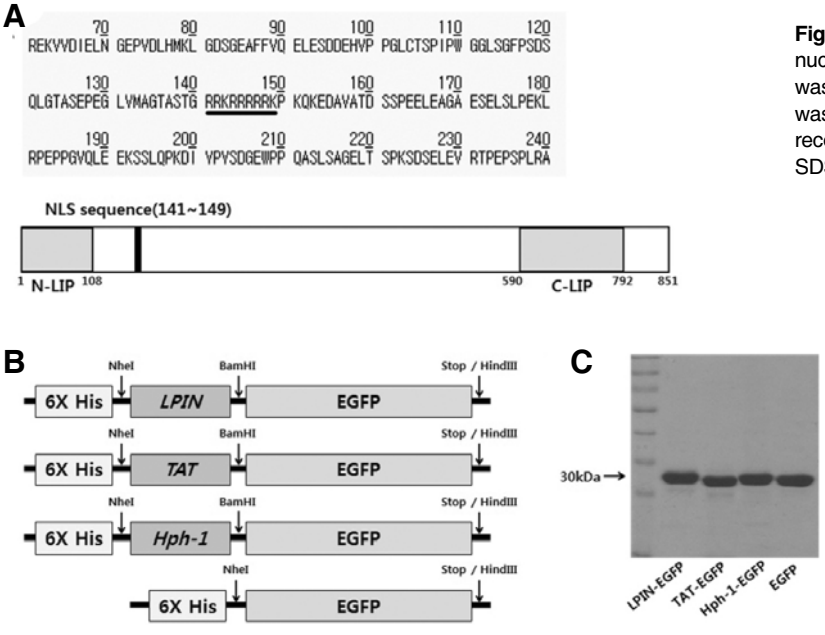

Fig. 1. Generation of CPP-EGFP proteins. (A) A nuclear localization sequence of LPIN3 (141-149) was selected as a candidate CPP. (B) Each DNA was inserted into a pRSET-B vector. (C) CPP-EGFP recombinant proteins were purified and analyzed by SDS-PAGE.

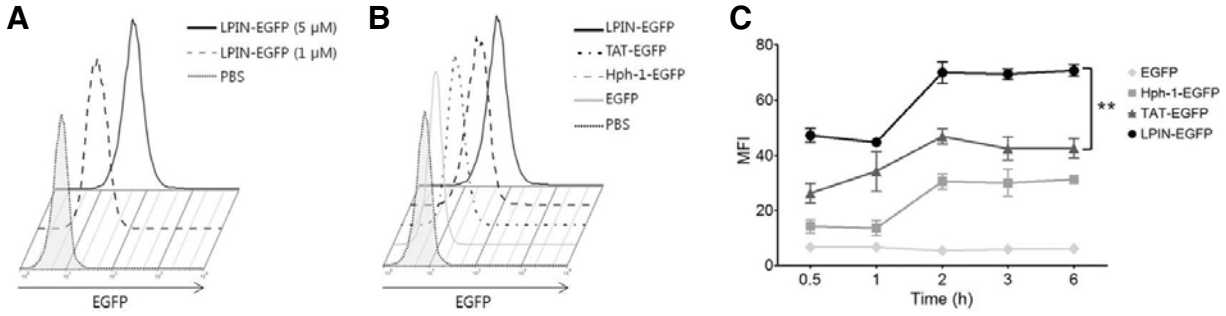

Fig. 2. Intracellular delivery efficiency of LPIN-EGFP. (A) The 1-5 μM LPIN-EGFP was added to Jurkat T cells for 2 h and protein delivery efficiency was analyzed by flowcytometry. (B) Delivery efficiency of LPIN- EGFP was compared with those of Hph-1-EGFP and TAT-EGFP. (C) Cell-penetrating kinetics of each CPP-EGFP protein was analyzed for 30 min (MFI, mean fluorescence intensity; $^{**}p < 0.005$, n = 3).

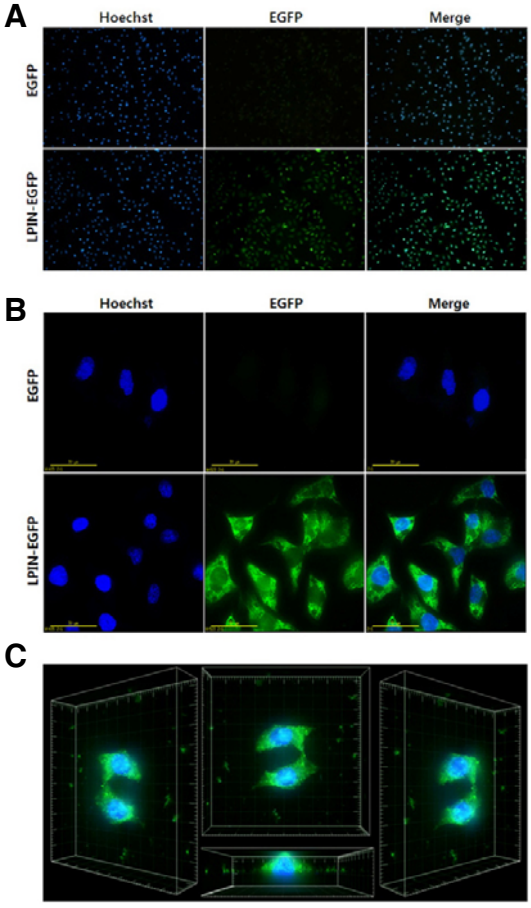

Fig. 3. Intracellular localization of LPIN-EGFP. HeLa cells were incubated with a 20 μM concentration of each protein for 2 h. Then cells were fixed and stained with Hoechst to visualize the nuclei. (A) Intracellular EGFP fluorescence was observed by fluorescence microscopy with X100 magnification. (B) Cells were visualized by confocal microscopy with X600 magnifi-cation. (C) The confocal micro-scopy images were merged and stacked into three-dimensional videos (captured images).

cellular delivery efficiency that was higher than that of EGFP without any CPP. In addition, transduction efficiency of LPIN-EGFP was higher than those of TAT-EGFP and Hph-1-EGFP at 5 μM, indicating that the nine amino acid-long LPIN-CPP has a stronger cell-penetrating ability than the 11 amino acids of TAT-CPP or Hph-1-CPP (Fig. 2B). Next, the transduction kinetics of each CPP-conjugated EGFP protein was analyzed. LPIN-EGFP was rapidly delivered into the cells within 30 min and the intracellular EGFP level was elevated for up to 2 h, similar to what was seen with TAT- and Hph-1-conjugated EGFP. However, LPIN-EGFP showed a much higher delivery efficiency than the other CPPs during all time points (Fig. 2C). These results suggest that the NLS of LPIN3 can be used as a novel cell-penetrating sequence to deliver various cargos, such as protein, and that its delivery efficiency is even better than those of the control CPPs including TAT-CPP and Hph-1-CPP.

Intracellular localization of LPIN-EGFP in HeLa cells

To examine intracellular delivery efficiency in another cell type and also to analyze its intracellular localization in the cells, 20 μM LPIN-EGFP was added to HeLa cells for 2 h and then the cells were stained with Hoechst to visualize nuclei. The delivery efficiency and intracellular localization were analyzed by confocal microscopy. Almost all cells could be seen to have taken up LPIN-EGFP while EGFP without CPP could not penetrate the cell membrane (Fig. 3A). Higher magnification (Fig. 3B) and 3-D imaging (Fig. 3C and Supplementary Video 1) showed that LPIN-EGFP was detected inside of vesicular compartments, cytoplasm and in the nucleus. These results suggest that the LPIN-CPP can deliver EGFP protein into the attached-type HeLa cells as well as the suspension-type Jurkat T cells and that LPIN-CPP can be used for delivery of not only cytosolic cargos but also of molecules intended for nuclear localization such as transcription factors, etc.

Cell-penetrating mechanism of LPIN-CPP

To determine whether the cell-penetrating mechanism of LPIN-CPP is energy-dependent, Jurkat T cells were treated with CPP-EGFP proteins at different temperatures (Fig. 4A). At 4°C, all CPPs including TAT-, Hph-1-, and LPIN-EGFP showed significantly reduced intracellular delivery efficiency, suggesting that LPIN-CPP has an energy-dependent cell-penetrating mechanism such as those of TAT and Hph-1. In order to confirm the molecular interaction between LPIN-CPP and cell membrane molecules, media with different serum contents were tested for delivery efficiency (Fig. 4B). As the serum percentage increased, the transduction efficiency of LPIN-EGFP was inhibited, suggesting that serum proteins could inhibit the interaction of

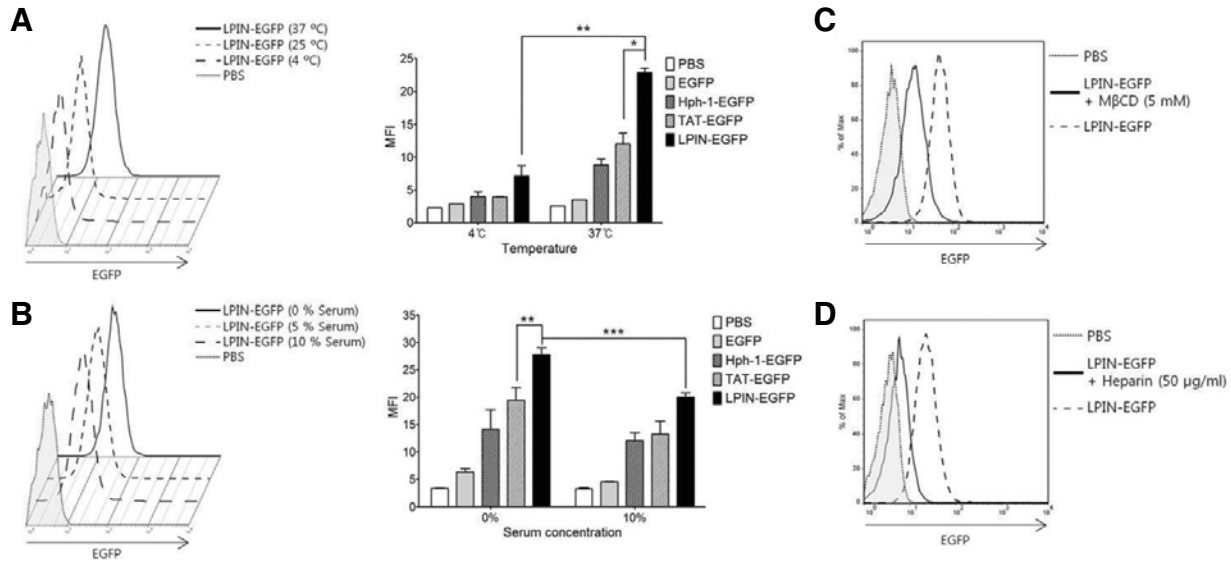

Fig. 4. Cell-penetrating mechanism of LPIN-EGFP. A 5 μM concentration of each protein was added to Jurkat cells for 2 h at different (A) temperatures or (B) concentration levels of serum-containing media. Intracellular fluorescence was analyzed by flowcytometry. Jurkat cells were pre-incubated with (C) MβCD for 20 min on ice or (D) heparin for 30 min at 37°C. Then, cells were treated with proteins for 1 h. (D) Delivery efficiency of LPIN-EGFP was analyzed by flowcytometry. All cells were washed with FACS buffer prior to analysis (*p < 0.05, **p < 0.005, ***p < 0.001, with each experiment performed in duplicate).

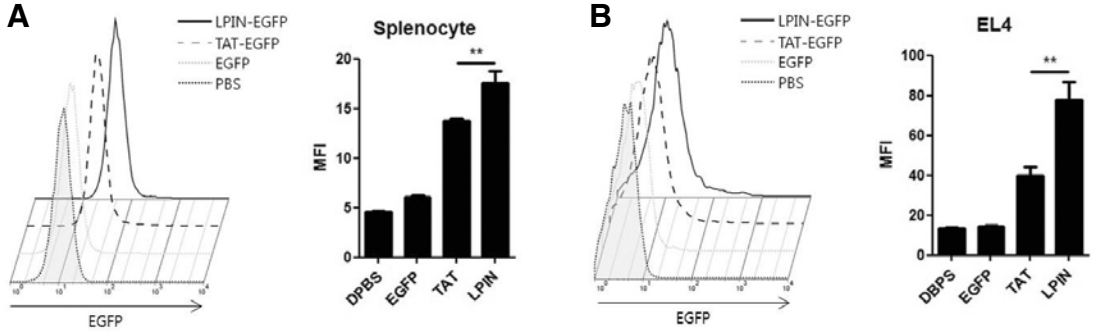

Fig. 5. Intracellular delivery of LPIN-EGFP into primary mouse cells. (A, B) Mouse splenocytes were isolated from C57bl/6 mice and 5 μM concentrations of each protein were added to these cells for 1 h. (C, D) EL4 mouse leukemia cells were treated with 5 μM concentrations of each protein for 1 h. Cells were harvested and washed before flowcytometric analysis (**p < 0.005, n = 3).

LPIN-CPP with cell membrane molecules. Next, the endocytosis inhibitor, methyl-beta-cyclodextrin (MβCD), (Fig. 4C) and a heparan sulfate interacting molecule of heparin (Fig. 4D) were used to better clarify the delivery mechanism of LPIN-CPP. The intracellular delivery efficiency of LPIN-EGFP was inhibited by MβCD, suggesting that lipid rafts mediate the endocytosis. Heparin treatment also strongly inhibited the intracellular delivery of LPIN-EGFP, suggesting that the initial interacting molecule on the cell membrane surface is heparan sulfate, *via* a charge interaction. These results suggest that LPIN-CPP interacts with heparan sulfate on the cell membrane and is then taken up by cells through lipid raft-mediated endocytosis.

Intracellular delivery of LPIN-EGFP into primary mouse cells

To confirm the cell-penetrating ability of LPIN-CPP and to prove its usefulness as a therapeutic delivery method *in vivo*, mouse splenocytes were isolated and treated with LPIN-EGFP (Fig. 5A). LPIN-EGFP could be significantly delivered into mouse primary cells, with its transduction efficiency being higher in EL4 mouse leukemia cells (Fig. 5B). These observations suggest that LPIN-CPP can deliver cargo into primary cells as well as cancer cells and indicate the possibility of using this system for successful cargo delivery *in vivo*.

***In vivo* delivery of LPIN-EGFP in mice**

To examine the *in vivo* cell-penetrating efficiency of LPIN-CPP, LPIN-EGFP protein was injected intra-peritoneally into mice and various organs including the liver, kidney, intestine, and brain. Tissues were then prepared as frozen slides for analysis by fluorescence microscopy (Fig. 6). LPIN-EGFP was rapidly delivered into the cells of the intestine within 1 h while increased fluorescence was detected at 2 h in the cells of liver and kidney, suggesting that *in vivo* administration of LPIN-EGFP could successfully deliver cargo proteins which need to be translocated into various tissues *in vivo*. However, delivery kinetics for each organ could be different and LPIN-CPP was not efficient for translocation into brain cells. These results suggest that LPIN-CPP, a novel human-originated cell-penetrating se-

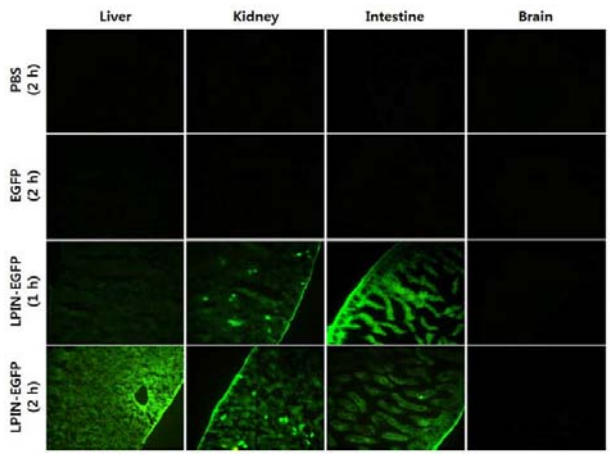

Fig. 6. *In vivo* delivery of LPIN-EGFP in mice. The mice were injected with LPIN-EGFP, EGFP, or an equivalent volume of PBS, intraperitoneally. After 1-2 h, all tissues were harvested and fixed and frozen slides were analyzed by fluorescence microscopy.

quence, makes it possible to deliver protein cargos into tissues *in vivo* and could be used for treatment of human diseases as a therapeutic delivery method.

DISCUSSION

LPINs are phosphatidate phosphatases that have multiple roles in the regulation of lipid metabolism and cell signaling (Donkor et al., 2007; Reue and Brindley, 2008). Most previous studies about LPINs involved lipid biosynthesis and its related roles in adipose tissue or, more recently, in macrophages (Valdearcos et al., 2011). Here, we have suggested a previously unknown function of amino acids derived from LPIN3 protein by identifying and characterizing a cell-penetrating peptide, which is located on its nuclear localization sequence.

For over 20 years, various amino acids sequences of CPPs have been developed including penetratin (Antp), polylysine, polyarginine, TAT, VP22, and pep-1 (Deshayes et al., 2005; Heitz et al., 2009; Zorko and Langel, 2005). Many therapeutic cargo molecules have been successfully delivered into cells via CPPs including proteins, peptides, si-RNA, DNA, and others (van den Berg and Dowdy, 2011; Zorko and Langel, 2005). TAT-BH4 and TAT-Bcl-XL inhibited sepsis-induced apoptosis of lymphocytes *in vivo* (Hotchkiss et al., 2006) and TAT-Cre was used to generate conditional knock-out mice (Wadia et al., 2004). Polyarginine (11R)-conjugated VIVIT peptide, which is a specific NFAT inhibitory peptide, was able to inhibit graft rejection of allogeneic islet transplantation in mice (Noguchi et al., 2004). More recently, TAT-CPP-conjugated RNA binding successfully delivered siRNA for efficient gene silencing in various cell types including primary T cells (Eguchi et al., 2009). Although extensive studies have suggested the possible use of those CPPs as drug delivery systems for human disease, clinical trials have been ongoing since the middle of the 2000s (van den Berg and Dowdy, 2011). However, because of their non-human origin, these CPPs carry the risk of possible immunogenicity and cytotoxicity. To overcome this limitation to therapeutic applications, the identification of a human-originated cell-penetrating peptide is quite valuable.

In our previous work, we identified a novel human-originated CPP, Hph-1, and showed that Hph-1-ctCTLA-4 recombinant protein inhibited T cell receptor signaling and demonstrated an inhibitory effect on allergic airway inflammation and autoimmune arthritis in animal models (Choi et al., 2006; 2008). LPIN-CPP is newly identified in this study and has nine amino acids, which is shorter than either TAT- or Hph-1-CPP. Furthermore, LPIN-CPP showed higher transduction efficiency than these control CPPs. There are several reports related to human-originated CPPs such as vactocell (De Coupade et al., 2005), lactoferrin (Duchardt et al., 2009), and others. More recently hC-CPP from the C-terminus of human extracellular superoxide dismutase was demonstrated to function as a human-originated CPP. However, most CPPs have more than 10 amino acids in their sequence (Zhao et al., 2011). Therefore, our novel human-originated LPIN-CPP could be the shortest CPP and also have a higher delivery efficiency.

Cationic amphiphilic peptides have been shown to bind to anionic lipid bilayers or cell surface heparan sulfate and then be taken up by endocytosis (Fischer et al., 2004). From previous reports, TAT-CPP was known to enter cells *via* an energy-dependent endocytic process. However, marginal amounts of proteins were still detected inside the cells even with a high concentration of inhibitors, suggesting the possibility of another transduction mechanism that might be used such as binding to sialic acid, macropinocytosis, or direct penetration. Because the NLSs of many proteins are composed of positive charge-rich sequences, it is likely that more human-derived CPPs will be identified through the analysis of NLSs as CPP candidates (Martin and Rice, 2007). These CPPs might also have a similar cell-penetrating mechanism and efficient intracellular delivery as LPIN-CPP.

Although a tremendous number of different CPPs have been previously reported, there are only a limited number of reports that have shown efficient *in vivo* delivery of cargo molecules (Choi et al., 2010; Heitz et al., 2009; Rittner et al., 2002; Schwarze et al., 1999). Because cancer cells express high levels of negatively charged molecules on the cell surface such as heparan sulfate, sialic acids, and phosphatidyl serine, CPPs have been studied mostly *in vitro* in cancer cell lines. Although most cells express heparan sulfate on their surfaces, the delivery efficiency of CPPs in primary cells is much weaker than that in cancer cell lines, suggesting that the amounts of negatively charged molecules in primary cells or *in vivo* tight junctions is a critical hurdle for the *in vivo* application of CPPs. LPIN-EGFP showed remarkable *in vivo* penetrating efficiency into various tissues including the liver, kidney, and intestine while LPIN-EGFP was not significantly detected in the brain at 1-2 h. This finding possibly suggests an increased difficulty for LPIN in penetrating the blood brain barrier (BBB).

As a drug delivery system for macromolecules in the development of bio-drugs, human-derived LPIN-CPP has an attractive therapeutic potential with a short amino acid sequence derived from a natural human protein without possible toxicity or immunogenicity. LPIN-conjugated therapeutic peptides, proteins, and RNAs will be further investigated by using disease models to develop therapeutic bio-drug candidates for human diseases.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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