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Cell-penetrating DNA-binding protein as a safe and efficient naked DNA delivery carrier *in vitro* and *in vivo*

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ABSTRACT

Non-viral gene delivery is a safe and suitable alternative to viral vector-mediated delivery to overcome the immunogenicity and tumorigenesis associated with viral vectors. Using the novel, human-origin Hph-1 protein transduction domain that can facilitate the transduction of protein into cells, we developed a new strategy to deliver naked DNA *in vitro* and *in vivo*. The new DNA delivery system contains Hph-1-GAL4 DNA-binding domain (DBD) fusion protein and enhanced green fluorescent protein (EGFP) reporter plasmid that includes the five repeats of GAL4 upstream activating sequence (UAS). Hph-1-GAL4-DBD protein formed complex with plasmid DNA through the specific interaction between GAL4-DBD and UAS, and delivered into the cells via the Hph-1-PTD. The pEGFP DNA was successfully delivered by the Hph-1-GAL4 system, and the EGFP was effectively expressed in mammalian cells such as HeLa and Jurkat, as well as in Bright Yellow-2 (BY-2) plant cells. When 10 μg of pEGFP DNA was intranasally administered to mice using Hph-1-GAL4 protein, a high level of EGFP expression was detected throughout the lung tissue for 7 days. These results suggest that an Hph-1-PTD-mediated DNA delivery strategy may be an useful non-viral DNA delivery system for gene therapy and DNA vaccines.

Introduction

Gene delivery has numerous potential applications in basic research and clinical uses including gene therapy and DNA vaccines. The efficient and non-toxic delivery of a target gene is a major concern of gene therapy researchers, but a satisfactory gene delivery system has not yet been found among viral and non-viral vectors. Viral vectors such as adenoviruses, retroviruses, and lentiviruses exhibit superior gene transfection efficiency but exert many adverse effects—including immunogenicity, toxicity, and mutagenesis—that limit their application [1]. Despite their low transfection efficiency, non-viral vectors have been extensively investigated as alternatives to viral vectors because of their advantages, which include low immunogenicity, no risk of mutational insertions, ease of preparation and modification, and unlimited delivery capacity for genetic material [2].

Protein transduction domains (PTDs) are short peptides that can penetrate the plasma membrane and deliver a variety of molecules in cells and animal models [3–6]. The HIV transactivator of transcription (TAT), the PTD of the *Drosophila* antennapedia transcription factor (Antp), a structural protein of herpes simplex virus (VP22), and polyarginine (R_n) have demonstrated enhanced DNA delivery efficiency *in vitro* [7–9]. TAT also enhanced liposome-mediated gene delivery *in vitro* and *in vivo*, although the PTD alone did not enhance plasmid uptake *in vivo* [10]. In a previous study, we identified a novel PTD (YARVRRRGPRR) from the human transcription factor Hph-1 and demonstrated its powerful protein transduction ability *in vitro* and *in vivo*, even through local administration routes [11].

The GAL4 DNA-binding domain (DBD), which consists of the N-terminal amino acids of the yeast transcription factor GAL4, possesses high binding affinity for a specific 17-bp oligonucleotide sequence and acts as a nuclear localization signal (NLS) [12]. These properties make it a good candidate for use as a gene transfer vehicle. Several groups have used the GAL4 DNA-binding domain to enhance gene delivery though conjugation of ligand and other cationic polymers [13–15]. However, cationic polymers such as poly-L-lysine and polyethyleneimine (PEI) can induce cytotoxicity and form aggregates in biological media containing plasma proteins, which limits their clinical application [16,17]. Thus, a non-toxic and efficient cell-penetrating DNA-binding protein would be an attractive option for gene delivery.

In the present study, we designed a new DNA delivery system consisting of the cell-permeable Hph-1-GAL4-DBD protein and

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DNA containing the upstream activating sequence (UAS). The Hph-1-GAL4 protein has a dual function: specific DNA binding via GAL4-DBD and the UAS of the DNA and intracellular delivery of the target DNA by Hph-1-PTD. This Hph-1-GAL4-mediated DNA delivery system effectively transduced the pEGFP DNA and induced EGFP expression in mammalian cells, and in BY-2 plant cells. Intranasal delivery of DNA/Hph-1-GAL4 complex resulted in a high level of EGFP expression in mouse lung tissue even at 7 days after delivery. These results demonstrate that our novel cell-penetrating DNA-binding protein holds great promise as a safe and effective non-viral gene carrier *in vitro* and *in vivo*.

Materials and methods

Materials. Escherichia coli BL21 star (DE3) pLysS and Lipofectamine™ 2000 were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Plasmids pEGFP-N1, pM, pG5CAT, and pRSET B were obtained from Clontech (Palo Alto, CA, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and RPMI-1640 were purchased from BioWhittaker (Walkersville, MA, USA). Modified Murashige and Skoog (MS) basal medium, polybrene, and chloroquine were obtained from Sigma–Aldrich (St. Louis, MO, USA), and YOYO-1 was purchased from Molecular Probes (Leiden, The Netherlands).

Cell culture. Human cervical cancer HeLa cells were grown in DMEM medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids. The Jurkat human acute T cell leukemia cell line was grown in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. Suspension-cultured tobacco BY-2 (*Nicotiana tabacum* L. cv BY-2) cells were grown in the dark at 26 °C on a rotary shaker (130 rpm) in modified MS basal medium supplemented with 1.5 mM KH_2PO_4, 3 μM thiamine, 0.55 mM inositol, 87 mM sucrose, and 1 μM 2,4-dichlorophenozy acetic acid. Cells were subcultured every 7 days by transferring 2 ml into 100 ml of fresh medium.

Construction and purification of Hph-1-fusion proteins. The GAL4-DBD (441 bp) that encodes amino acids 2 through 147 of the yeast transcription factor GAL4 was amplified from the pM plasmid by polymerase chain reaction (PCR). It was subsequently cloned into the bacterial recombinant protein expression vector pRSET B. The Hph-1-GAL4 protein contained the coding sequence for Hph-1-PTD (YARVRRRGPRP) at its N-terminus. The EGFP gene was inserted between the Hph-1 and GAL4 sequences to generate the Hph-1-EGFP-GAL4 protein for cellular uptake research. GAL4 and EGFP-GAL4 proteins lacking Hph-1-PTD were also constructed and purified for use as negative controls.

The protein expression vectors were transformed into *E. coli* BL21 star (DE3) pLysS cells and cultured in Luria–Bertani (LB) broth medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Protein expression was induced for 4 h at 37 °C by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in lysis buffer (10 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0), and lysed by sonication. Lysates were clarified by centrifugation (13,000 rpm for 20 min at 4 °C) and gently mixed with 50% slurry of Ni–NTA resin (Qiagen, Hilden, Germany) for 1 h at 4 °C. Unbound proteins were removed by washing the column with 20 mM imidazole buffer; bound proteins were eluted with 250 mM imidazole buffer. Proteins were dialyzed against 10% glycerol in phosphate buffered saline (PBS).

Target DNA construction. The pEGFP-UAS, containing the GAL4 upstream activating sequence (UAS; CGGAGGACAGTACTCCG) and the gene encoding the enhanced green fluorescent protein, was constructed using plasmid pEGFP-N1. The GAL4-specific site was amplified from a pG5CAT vector containing five consensus GAL4 binding sites (UAS17merx5). For the study of DNA transfection in the Nicotiana tabacum BY-2 cell line, five consensus GAL4 binding sites (UAS17merx5) were cloned into the pSMGFP vector.

Gel retardation assay. To confirm DNA condensation and binding ability of the Hph-1-GAL4 protein, plasmid DNA was incubated with Hph-1-GAL4 protein according to indicated amine/phosphate (N/P) ratios for 15 min at room temperature and then electrophoresed on 0.7% agarose gel. The DNA was visualized using an UV illuminator after ethidium bromide (EtBr) staining.

Cytotoxicity assay. Cytotoxicity of the Hph-1-GAL4 protein was determined using a cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). HeLa cells were seeded into 96-well plates (1×104 cells/well) and incubated for 24 h before experiments. Cells were incubated with 0.1–50 μM Hph-1-GAL4 protein for 3 h and then exposed to WST-8 reagent for 4 h at 37 °C in a 5% CO_2 incubator. Cell viability was determined by measuring absorbance at 450 nm using a microplate reader (Molecular Devices, CA, USA). Data are expressed as means ± standard deviation (SD) of three measurements.

Transduction of the Hph-1-fusion protein. To monitor the concentration-dependant and time-dependant transduction of the Hph-1-PTD-fusion protein, HeLa cells were incubated with increasing concentrations of Hph-1-EGFP-GAL4 protein for 2 h, and 2 μM Hph-1-EGFP-GAL4 protein was added to HeLa cells at various time points. After incubation, cells were washed extensively with PBS, and fluorescence was detected by FACSCalibur (BD Bioscience, CA, USA). EGFP-GAL4 lacking Hph-1-PTD was used as a negative control.

Cellular uptake of DNA. To confirm cellular uptake of plasmid DNA by Hph-1-GAL4 protein, DNA was labeled with YOYO-1, a DNA-intercalating dimeric cyanine fluorescent dye. YOYO-1-labeled DNA (3 μg) was mixed with 10 μM Hph-1-GAL4 protein at room temperature for 15 min and then incubated with HeLa and Jurkat cells in 6-well plates for 2 h. Surface-bound DNA was then washed with PBS, and fluorescence was quantified by flow cytometry. Cells treated with YOYO-1-labeled DNA alone, without any PTD-fusion protein, were used as a control.

In vitro transfection. DNA was mixed with Hph-1-GAL4 at room temperature for 15 min, after which HeLa and Jurkat cells were incubated with this mixture under serum-free conditions for 3 h. In Jurkat cells, polybrene was also added to the DNA/protein complex to enhance transfection efficiency [18]. The serum-free medium was replaced with complete medium after incubation, and transgene expression was analyzed by flow cytometry 48 h later. We compared the transfection efficiency of Hph-1-GAL4 with those of Lipofectamine™ 2000 and TAT-PTD. To determine whether facilitated escape from endosomes could enhance the transfection efficiency of PTD-fusion proteins, 200 μM chloroquine was used as an endosomal disruption reagent.

After BY-2 cells were seeded into 6-well plates, transfection was performed as described above for mammalian cells. GFP fluorescence was determined using a fluorescence microscope (Axio Imager, Carl Zeiss, Germany).

In vivo transfection. To investigate the transfection efficiency of the Hph-1-GAL4 protein *in vivo*, a DNA/protein complex (10 μg and 250 μg, respectively) was intranasally administered to anesthetized 6-week-old male BALB/c mice (Samtaco, Korea). After 3 and 7 days, lungs were separated, embedded in OCT compound, frozen, and cut into 0.5-μm sections. Sections were mounted on slides, and EGFP expression was directly observed with a fluorescence microscope (ECLIPSE TE2000-U; Nikon, Japan).

Results and discussion

Generation of a cell-penetrating DNA delivery system

To develop a more efficient non-viral DNA delivery system, we designed a new cell-permeable form of DNA-binding protein that contained the novel, human-origin Hph-1-PTD (YARVRRRGPRR) and GAL4 DNA-binding domain (DBD). GAL4-DBD possesses a high affinity for a specific 17-bp upstream activation sequence (UAS). This enables the Hph-1-GAL4 protein to specifically bind target DNA containing the UAS and deliver it into cells via the Hph-1-PTD (Fig. 1). Each UAS can interact with one GAL4 protein dimer; thus, the insertion of more than one UAS may help promote efficient binding. Therefore, we constructed an EGFP expression plasmid that included five copies of the UAS. The Hph-1-PTD sequence was inserted in the N-terminus of GAL4-DBD, and the EGFP sequence was fused between Hph-1 and GAL4-DBD (Fig. 2A). We also generated GAL4 and EGFP-GAL4 without Hph-1-PTD for use as negative controls. Using the *E. coli* recombination protein expression system, pure proteins were obtained under native conditions, and purity was assessed by Coomassie brilliant blue staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2B).

Formation of DNA/protein complex and cell viability

DNA-binding ability is a prerequisite for an ideal gene delivery vector. To confirm whether Hph-1-GAL4 protein binds to pEGFP-UAS DNA *in vitro*, a gel retardation assay was performed at various amine/phosphate (N/P) ratios. As the N/P ratio was increased, DNA mobility was retarded, and mobility was completely stopped at an N/P ratio of 20. This indicates that Hph-1-GAL4 protein can condense negatively charged DNA (Fig. 2C). On the basis of these results, the N/P ratio was fixed at 20 or above for further transfection assays.

A CCK-8 assay was performed to evaluate the potential toxicity of the Hph-1-GAL4 protein. In results consistent with our previous study, the Hph-1-PTD-fusion protein did not affect cell viability [11,19]. Hph-1-GAL4 protein did not produce significant cytotoxicity even at high concentrations; 50 μM Hph-1-GAL4 protein reduced cell viability slightly, but cell viability remained greater than 90% (Fig. 2D). This result shows that Hph-1-GAL4 protein can be used as a non-toxic gene carrier.

Delivery of Hph-1-fusion protein and pEGFP-UAS DNA

To investigate whether the PTD-fusion protein itself can directly penetrate cells, we monitored transduction of the Hph-1-EGFP-GAL4 protein, which contained the EGFP sequence between Hph-1 and GAL4. HeLa cells were incubated with varying concentrations of Hph-1-EGFP-GAL4 protein for 2 h. Then, the cells were washed, and EGFP expression was analyzed by flow cytometry (Fig. 3A). Hph-1-EGFP-GAL4 produced a strong green fluorescence signal in a concentration-dependent manner. In contrast, 10 μM EGFP-GAL4 lacking Hph-1-PTD produced no fluorescence. To determine the time-dependent transduction kinetics of the Hph-1-fusion protein, HeLa cells were incubated with 2 μM Hph-1-EGFP-GAL4 protein for 30 min to 6 h, after which the cells were washed and EGFP expression was analyzed (Fig. 3A). The Hph-1-fusion protein penetrated cells in a time-dependent manner. The control EGFP-GAL4 protein lacking Hph-1-PTD did not penetrate cells, even at 6 h after transduction (Fig. 3A).

To demonstrate that plasmid DNA can be delivered into cells by the Hph-1-GAL4 protein, we labeled the pEGFP-UAS DNA with YOYO-1 dye—a DNA-intercalating reagent. HeLa and Jurkat T cells were incubated with YOYO-1-labeled DNA and Hph-1-GAL4 protein complexes, and transduction was analyzed by flow cytometry after 3 h. pEGFP-UAS DNA was efficiently delivered into both cells, but cells treated with naked DNA alone did not produce a fluorescence signal (Fig. 2B). These results show that the Hph-1-PTD can efficiently deliver the covalently conjugated GAL4 protein, as well as the non-covalently associated DNA, into the cells.

In vitro transfection efficiency

To investigate whether the delivered pEGFP-UAS DNA can be expressed in mammalian cells, HeLa cells were incubated with a mixture of pEGFP-UAS and protein (Hph-1-GAL4 or GAL4), and the level of EGFP expression was analyzed by flow cytometry. HeLa cells transfected with Hph-1-GAL4 showed substantially higher

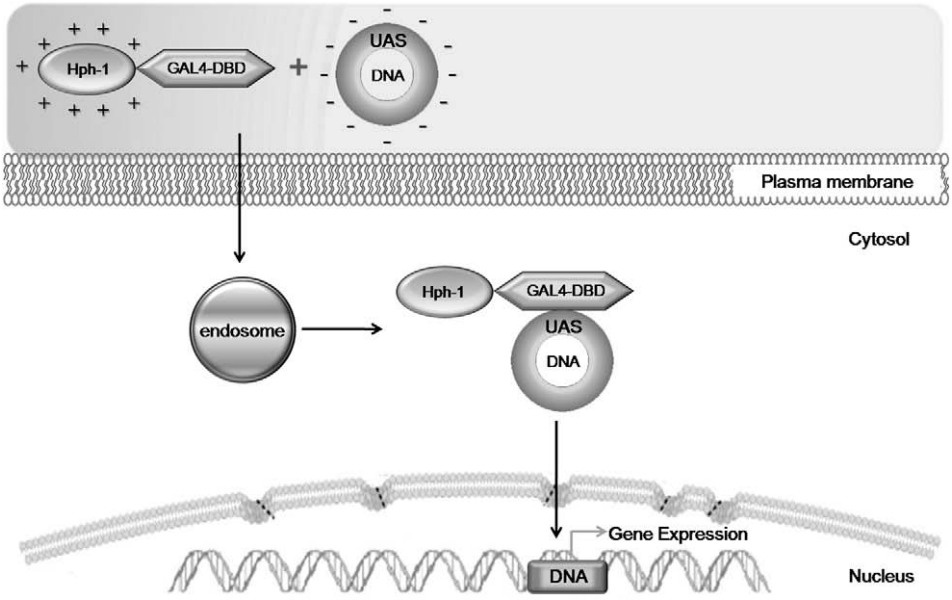

Fig. 1. Schematic of Hph-1-GAL4-mediated DNA delivery system.

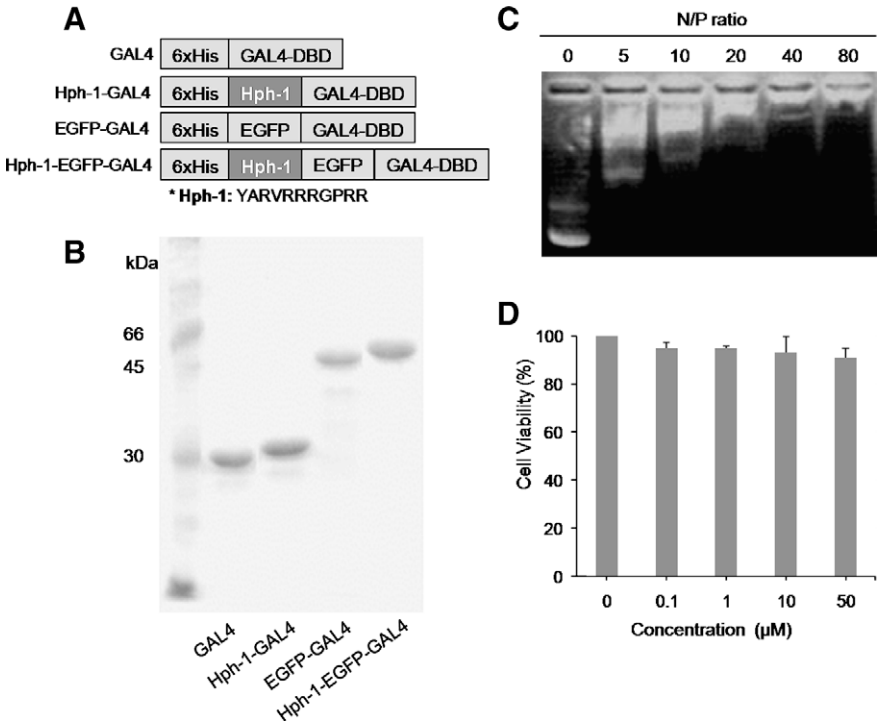

Fig. 2. Purification and characterization of Hph-1-fusion proteins. (A) Construct of Hph-1-PTD-fusion proteins. (B) Purified Hph-1-PTD-fusion proteins were separated by SDS–PAGE (10%) and stained with Coomassie brilliant blue. (C) Gel retardation assay was performed at various N/P ratios. (D) HeLa cells were incubated with a various concentrations of Hph-1-GAL4 protein for 3 h, and cytotoxicity was determined by a CCK-8 kit.

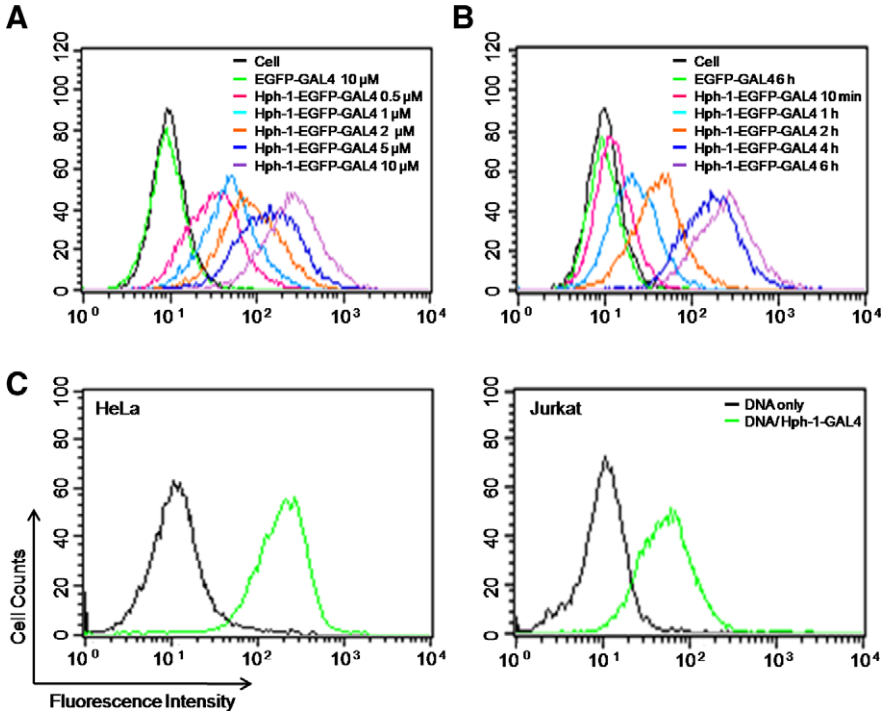

Fig. 3. Transduction of Hph-1-fusion protein and DNA. (A) HeLa cells were incubated with increasing concentrations of Hph-1-EGFP-GAL4 for 2 h or with 2 μM Hph-1-EGFP-GAL4 for 30 min to 6 h (B). Enhanced green fluorescent protein (EGFP) expression was determined by fluorescent-activated cell sorting (FACS) analysis. (C) Hph-1-GAL4 efficiently delivered YOYO-1-labeled DNA into HeLa and Jurkat cells.

fluorescence (Fig. 4A), and the efficacy of Hph-1-PTD-mediated DNA delivery was concentration-dependent (Fig. 4B). However, cells treated with GAL4 protein lacking Hph-1 did not show fluorescence (Fig. 4A). Next, HeLa cells were transfected with Hph-1-GAL4 protein and either pEGFP-N1 or pEGFP-UAS DNA. Cells transfected with pEGFP-N1 lacking the GAL4-specific binding site exhibited low fluorescence (Fig. 4C) because of electrostatic interactions with the positively charged Hph-1. The pEGFP-UAS group showed

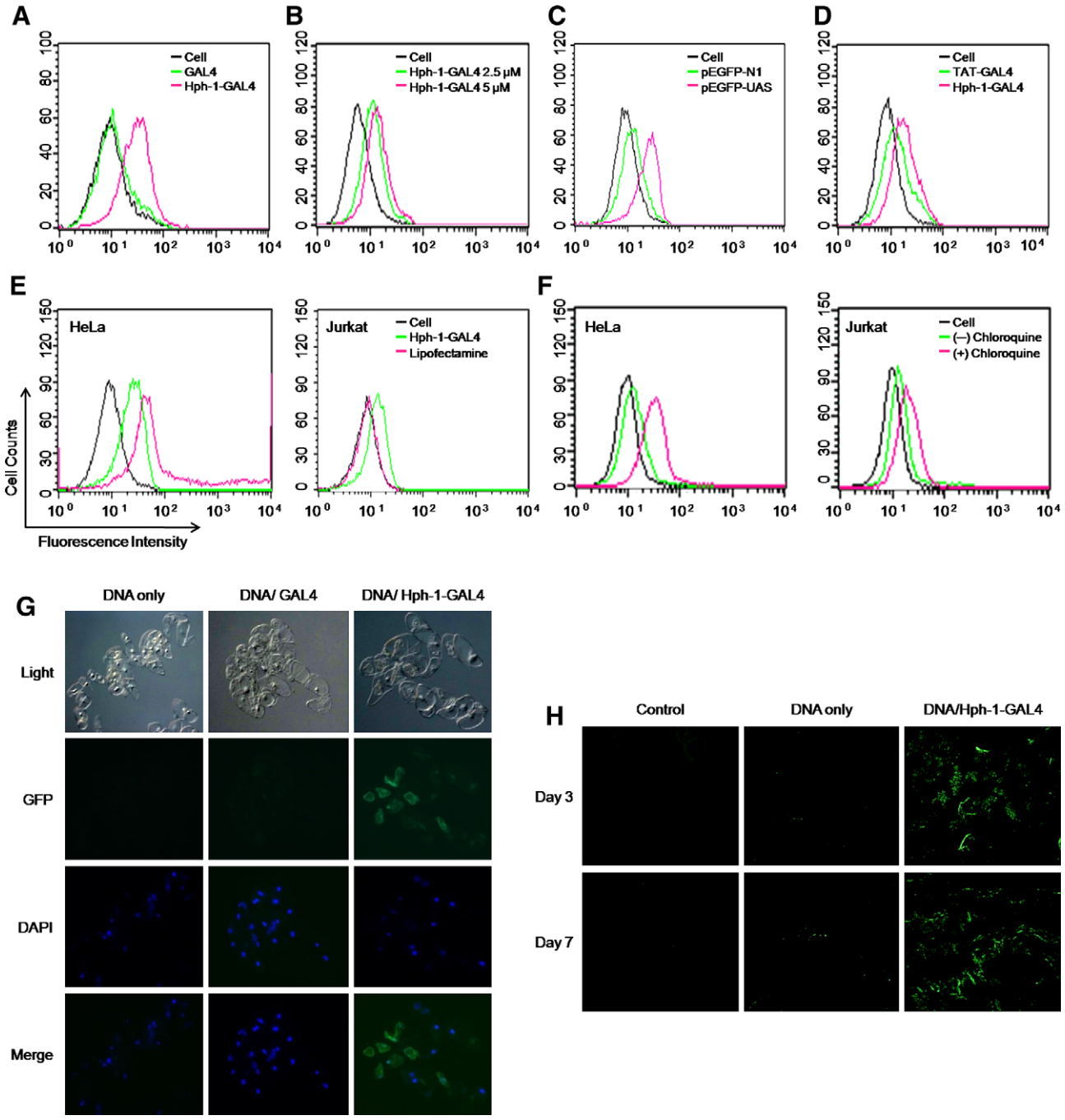

Fig. 4. *In vitro and in vivo* transfection. (A) pEGFP-UAS DNA was transfected with Hph-1-GAL4 or GAL4 lacking Hph-1-PTD in HeLa cells. (B) Transfection efficiency was analyzed with different concentrations of Hph-1-GAL4 protein. (C) pEGFP-N1 DNA lacking the GAL4 upstream activating sequence (UAS) showed relatively low transfection efficiency compared to pEGFP-UAS DNA in HeLa cells. (D) Hph-1-GAL4-mediated transfection was compared with TAT-GAL4-mediated transfection and (E) Lipofectamine-mediated transfection. (F) Transfection efficiency was enhanced by chloroquine in HeLa and Jurkat cells. (G) Hph-1-GAL4-mediated DNA transfection was assessed in BY-2 cells by fluorescence microscopy. (H) pEGFP-UAS/Hph-1-GAL4 complex was intranasally administered to mice. EGFP expression in the lung tissue was determined by fluorescence microscopy 3 and 7 days later.

strong fluorescence, indicating that Hph-1-GAL4 specifically bound and delivered DNA containing the UAS into cell nuclei where gene transcription occurred.

TAT-PTD derived from 11 amino acids (YGRKKRRQRRR) of HIV TAT protein has been widely used for the delivery of various molecules *in vitro* and *in vivo* [3–7]. We constructed and purified the TAT-GAL4 protein as a typical PTD for comparison of transfection efficiency with Hph-1-GAL4. Cells incubated with Hph-1-GAL4 showed stronger fluorescence than did TAT-GAL4-treated cells (Fig. 4D). Youn et al. reported that Hph-1 enhanced the transfection efficiency of adenovirus-mediated GFP gene delivery and produced more GFP-positive cells than did TAT-PTD [20].

We then compared the transfection efficiency of Hph-1-GAL4 to that of Lipofectamine, which is the most common reagent used to introduce DNA into cells. In HeLa cells, Lipofectamine produced transfection efficiencies similar or slightly higher than those of Hph-1-GAL4. However, Hph-1-GAL4 produced higher EGFP expression than did Lipofectamine in Jurkat cells (Fig. 4E). Jurkat cells are

grown in suspension culture, and it is very difficult to introduce DNA and express transgenes in these cells with a commercial lipofection reagent. Lipofection is a convenient reagent, but its transduction efficiency depends largely on the cell types and experimental conditions used. Additionally, lipofection has been shown to cause the death of 35–65% of cells in a concentration-dependent manner [10]. Furthermore, the introduced nucleotides are not specifically directed to the nucleus; thus, they remain in the cytoplasm after lipofection *in vitro* and *in vivo* [21,22]. Our findings demonstrate that Hph-1-GAL4 protein can deliver pEGFP-UAS DNA and efficiently express the target gene in all types of cells, including cells like Jurkat cells grown in suspension culture.

Gene delivery and expression is a multiple-step process: compacting DNA into the particle, delivering DNA into target cells and tissues, release of DNA from the endosome into the cytoplasm, and nuclear localization and transcription. YOYO-1-labeled DNA penetrated cells successfully, but the gene expression level was lower than expected (Figs. 3B and 4A–E). Although little is known regarding the mechanism by which PTD-conjugated macromolecules are released from endosomal vesicles, the transfer of PTD-conjugated cargo from the endosome to the cytosol has been suggested to be the rate-limiting step of PTD-mediated transduction [23–25]. To determine whether endosomal trapping of the DNA/PTD complex affected transfection efficiency, we added chloroquine (endosomolytic agent) to the culture medium in which HeLa and Jurkat cells were incubated with the pEGFP-UAS/Hph-1-GAL4 complex. *EGFP* gene expression was enhanced in the presence of chloroquine (Fig. 4F), as was previously observed [7,26]. This suggests that endosomal escape is required to increase the efficiency of gene delivery via a PTD-mediated system.

In basic plant research, plant biotechnology, and molecular farming, *Agrobacterium*-based transformation is the most widely used technology for gene transfer [27]. Several non-*Agrobacterium* bacterial species and virus-based vectors have also been investigated as alternatives to *Agrobacterium* [27]. The tobacco BY-2 cell line is a powerful system to study plant cells because it can proliferate up to 100-fold within 1 week and produces relatively homogeneous cultures [28]. To investigate the utility of Hph-1-GAL4-mediated gene delivery in plant cell transformation, we transduced the plasmid pSMGFP-UAS into BY-2 cells using Hph-1-GAL4. Transfection was performed with the same method used for mammalian cell transfection, and GFP expression was detected by fluorescence microscopy. Cells treated with Hph-1-GAL4 showed a strong fluorescence signal, but cells treated with GAL4 lacking the PTD and or naked DNA alone did not fluoresce (Fig. 4G). We therefore believe that a PTD-mediated DNA delivery system may be an useful and time-saving alternative to *Agrobacterium* in plant cell transformation.

In vivo transfection efficiency

To determine the efficiency of Hph-1-GAL4-mediated gene delivery *in vivo*, the pEGFP-UAS/Hph-1-GAL4 complex was intranasally administered to BALB/c mice. In addition to its use for local drug delivery in cases such as nasal allergy and infection, intranasal administration has recently been investigated for the systemic delivery of small drugs not easily administered via other routes [29]. However, the structure and function of the lung system are complex, and the lungs' thick mucus lining is a barrier to pulmonary drug delivery [30]. Thus, the development of a safe and efficient DNA delivery vector for intranasal administration may open many possibilities for medical research and clinical applications. pEGFP-UAS (10 μg) was mixed with Hph-1-GAL4 protein (250 μg) for 15 min at room temperature, and the complex was intranasally injected into the mice. After 3 and 7 days, EGFP expression of lung was measured by fluorescence microscopy. The group treated with Hph-1-GAL4 showed strong fluorescence throughout the entire lung tissue, but naked DNA produced little fluorescence signal (Fig. 4H). In the present study, 10 μg of DNA was sufficient to express target DNA in the mouse lung, and the expression was maintained for 7 days with a single injection. This suggests that the Hph-1-mediated DNA carrier system holds great promise as a gene delivery vector to treat respiratory diseases and deliver DNA vaccines.

Conclusion

We designed a novel DNA delivery vehicle containing Hph-1-PTD and GAL4-DBD. GAL4-DBD is capable of binding DNA containing its specific binding sequence, and Hph-1-PTD is able to penetrate cell membranes to deliver DNA. We demonstrated the successful delivery and expression of the reporter gene EGFP in mammalian cells and plant cells using this system. Furthermore, intranasal delivery of the DNA/Hph-1-GAL4 complex resulted in strong gene expression in mouse lung tissues. Thus, our cell-penetrating DNA-binding protein may be an useful and safe non-viral gene delivery carrier.

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