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The third helix of the murine Hoxc8 homeodomain facilitates protein transduction in mammalian cells

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ABSTRACT

Previously, we have demonstrated that purified Hoxc8 homeoprotein has the ability to penetrate the cellular membrane and can be transduced efficiently into COS-7 cells. Moreover, the Hoxc8 protein is able to form a complex with DNA molecules in vitro and helps the DNA be delivered intracellularly, serving as a gene delivery vehicle. Here, we further analyzed the membrane transduction activity of Hoxc8 protein and provide the evidence that the 16 amino acid (a.a.191–206, 2.23 kDa) third helix of murine Hoxc8 protein is an efficient protein transduction domain (PTD). When the 16 amino acid peptide was fused at the carboxyl terminal of enhanced green fluorescence protein (EGFP), the fusion proteins were transduced efficiently into the primary pig fetal fibroblast cells. The transduction efficiency increased in a concentration-dependent manner up to 1 μM, and appeared to plateau above a concentration of 1 μM. When tandem multimers of PTD, EGFP-PTD(2), EGFP-PTD(3), EGFP-PTD(4), and EGFP-PTD(5), were analyzed at 500 nM of concentration, the penetrating efficiency increased in a dose-dependent manner. As the number of PTDs increased, the EGFP signal also increased, although the signal maintained plateau after EGFP-PTD(3). These results indicate that the 16 amino acid third helix is the key element responsible for the membrane transduction activity of Hoxc8 proteins, and further suggest that the small peptide could serve as a therapeutic delivery vehicle for large cargo proteins.

Homeoproteins belong to a family of transcription factors that share the evolutionary conserved DNA-binding domain called the homeodomain [1]. In addition to their transcriptional activities, a few members of the family such as *Drosophila* Antennapedia and mammalian Hoxa5 have been shown to have the ability to transverse the cellular membrane and be internalized intracellularly in vitro [2,3]. Even though the mechanism of the protein internalization is not clear [4], the reports on Antennapedia suggest that the internalization is mediated by binding of these proteins to the cell surface through electrostatic interactions, followed by formation of the inverted micelles that can entrap and deliver the proteins to the cytoplasm [5]. Antennapedia has a 60 amino acid homeodomain, which consists of three helices, and mutagenesis experiments revealed that the third helix of the homeodomain appears to be responsible for the membrane transducing ability of the protein [3]. In particular, a small amino acid domain in the third helix called protein transduction domain (PTD) has been shown to be sufficient to induce the internalization of the proteins [6]. In addition, the PTDs are capable of forming complexes with and delivering high molecular weight molecules such as polypeptides and nucleic acid into cells [4]. Since this internalization process seems to be non-endocytic, non-degradative, and energy-independent [7], it raised the possibility that the PTDs can serve as a therapeutic delivery vehicle for cargo molecules into the cells [8]. In fact, there have been a few reports showing small peptides exhibiting similar transduction activities to deliver macromolecules into the cells in vitro and in vivo [9–13].

Hoxc8 is a homeodomain protein and acts as a transcriptional regulator in a variety of developmental processes during embryogenesis [14]. We noticed that the homeodomain of murine Hoxc8 is highly homologous with the *Drosophila* Antennapedia homeodomain. In particular, the third helix of the Hoxc8 homeodomain possesses 94% homology with that of Antennapedia. Consistent with the sequence homology, we have previously demonstrated that similar to the *Drosophila* Antennapedia proteins, murine Hoxc8 is also able to penetrate the cellular membrane and efficiently internalized into the cells [15]. In addition, when the entire Hoxc8 protein was fused with EGFP, the fusion proteins were efficiently transduced into COS-7 cells in vitro [15]. Furthermore, it has also been shown that Hoxc8 proteins can form complexes with DNA molecules in vitro and deliver them into the cells [16].

Here, we further characterized the membrane transducing ability of Hoxc8, and demonstrate that 16 amino acid peptide (a.a.191–206) of the third helix in Hoxc8 homeodomain can act

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as an efficient protein transduction domain (PTD). EGFP fusion proteins with the 16 amino acid peptide were internalized efficiently into the primary pig fetal fibroblast cells. The transduction efficiency of the EGFP-PTD fusion protein was concentration-dependent, and was also enhanced when multiple copies of the PTD peptide were fused. Our results indicate that the 16 amino acid peptide is the key element responsible for the membrane transducing ability of Hoxc8, and may serve as a therapeutic delivery vehicle for macromolecules such as proteins and nucleic acids.

Materials and methods

Plasmid constructs. Oligonucleotides encoding the 16 amino acid PTD domain ($Hoxc8_{191-206}$) were synthesized (Genotech, Korea); 5′-gatct cgcca ggtga aagtt tggtt tcaga accgc cgcat gaaat ggaaa aaag-3′ and 3′-agcgg tccac tttta aacca aagtc ttggc ggcgt acttt acctt ttttc ctag-5′. *Bgl*II and *Bam*HI sites were added at the 5′ and 3′ termini, respectively, for cloning and multimerization. The enhanced green fluorescent protein (EGFP) gene digested from pEGFP-C1 with NcoI and BamHI was first cloned into the bacterial expression vector, pET-32a (+) (Novagen). Then, the 54-mer oligonucleotides encoding the 16 amino acid Hoxc8 PTD region were cloned into the BamHI site of pET-EGFP vector, named pET-EGFP-PTD(1). Taking the advantage of the same sticky end (GATC) of both 5′ BglII and 3′ BamHI, multiple PTD copies were inserted into pET-EGFP orderly in correct orientation (tandem head to tail direction).

Expression and purification of recombinant proteins. Fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells. For induction of the recombinant proteins, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the bacterial culture and the cells were harvested 4 h later. The cells were resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), which contains lysozyme (1 mg/ml), for 30 min on ice. Then, the cells were disrupted by sonication and pelleted at 10,000*g*. The supernatant was mixed with Ni/NTA (Quiagen) and incubated for at least 1 h whilst gentle shaking at 4 °C. The material was transferred into a column and washed with washing buffer (50 mM sodium phosphate, 300 mM NaCl, 40 mM imidazole pH 8.0) and the bound protein eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 8.0). The apparent size and the purity of the product were examined by SDS–PAGE and the concentration was estimated by standard Bradford assay.

Cell culture and protein transduction. Porcine primary fetal fibroblast cells (PPFF) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Carlsbad, CA) containing 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cells (2×10^4/well of a 12-well culture plate) cultured overnight were rinsed and incubated in fresh DMEM containing PTD fusion proteins at different concentrations (50 nM to 2 μM) for 48 h. The cells were fixed with 4% paraformaldehyde, and permeabilized with absolute methanol, and then nuclei were stained with Hoechst. Fluorescent signals were observed by a fluorescence microscope (Olympus IX70: Olympus, Melville, NY).

Flow cytometry. For flow cytometry analysis, cells (2×10^4 cells/well in a 12-well plate) were grown overnight at 37 °C. To these cells different concentration of purified recombinant EGFP fusion PTD (~80%) was added and incubated for 48 h at 37 °C. The cells were washed with 1× PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), detached from the plates with 0.01% trypsin and 10 mM EDTA in PBS, washed in PBS, and used directly for flow cytometric analysis. Internalization of the fusion proteins was determined in a FACS calibur flow cytometer (Dickinson). Approximately 10,000 cells were analyzed per data point, and all experiments were carried out in triplicate.

Results and discussion

Expression and purification of recombinant protein

In order to test whether the third helix of the murine Hoxc8 homeodomain has membrane traversing activity, oligonucleotides encoding the 16 amino acid long peptide (Hoxc8 a.a.191–206 protein transduction domain, PTD) were synthesized based on *E. coli* codon usage (Fig. 1A) and then cloned into the high-level expression plasmid vector pET-32a(+) together with the green fluorescent protein (EGFP) gene in frame. Since this vector is supposed to express the PTD as a fused protein of Trx-His-S-EGFP-PTD, the recombinant fusion protein was expected to be solubilized better in *E. coli* because of the thioredoxin (Trx) tag and purified more easily because of the 6 histidine (His) residue tag. Furthermore, EGFP makes the recombinant fusion protein readily detectable under the fluorescence microscope. Plasmids expressing multi copies of PTD were also constructed, as described in Materials and methods (Fig. 1B). To overexpress the fusion protein, *E. coli* strain BL21 (DE3) was transformed with each of the plasmid encoding EGFP only,

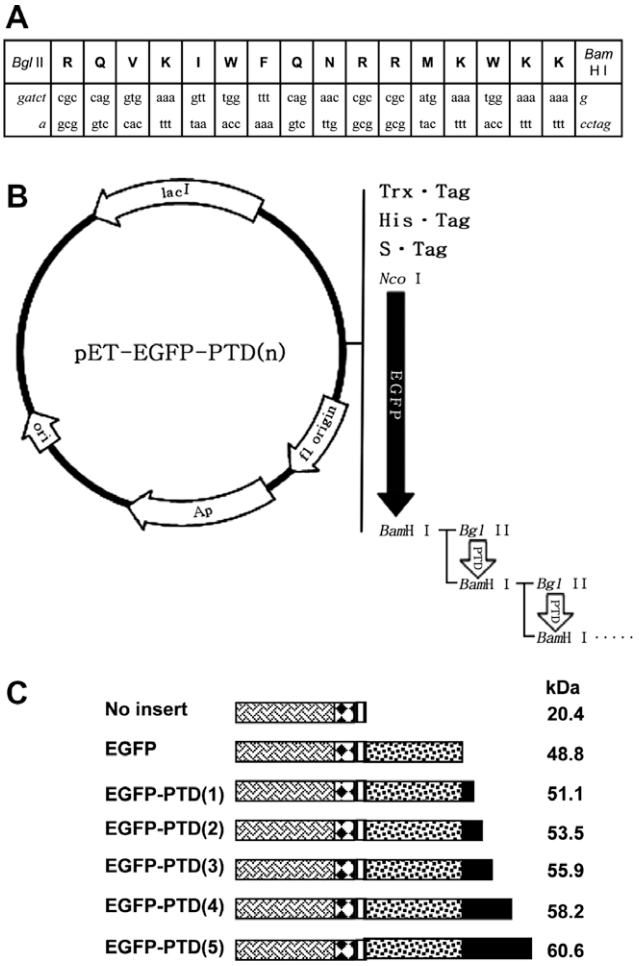

Fig. 1. Constructs expressing EGFP-PTD fusion proteins. (A) The amino acid sequence of the third helix within the homeodomain of murine Hoxc8 protein, and the corresponding oligonucleotide sequences encoding the 16 amino acids. (B) Schematic diagram of the plasmid pET-EGFP-PTD(n) expressing EGFP-PTD fusion proteins. The insertion sites and the direction of the coding sequences of EGFP and PTD are indicated as arrows. Trx tag (thioredoxin protein, 109 a.a.), His tag (6 Histidines), and S protein tag were also indicated. (C) Structures of the fusion proteins. Trx tags are indicated by weaved boxes, His tags with solid diamond texture, S protein tags with slashed boxes, EGFP with dotted boxes, and finally PTDs with dark boxes. Expected sizes of each recombinant fusion protein are shown in kDa.

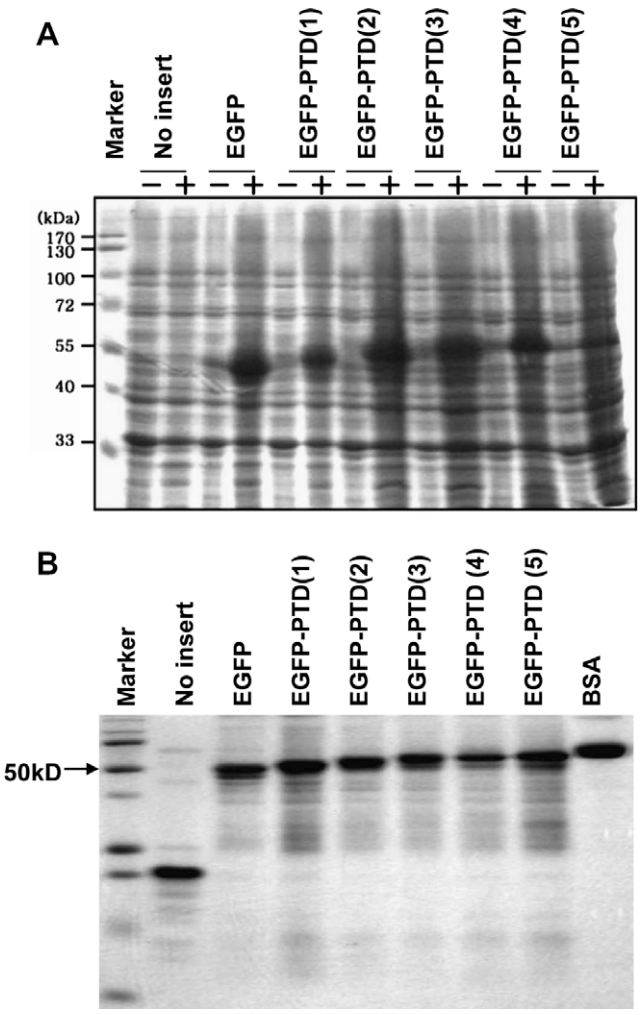

Fig. 2. Expression and purification of EGFP-PTD fusion proteins. (A) SDS–PAGE with the whole lysates of *E. coli* transformed with each plasmid containing pET-EGFP, EGFP-PTD(1), -PTD(2), -PTD(3), -PTD(4), or -PTD(5). Strong expressions of the fusion proteins were evident at around 50 kD only in the bacterial cultures with IPTG induction. (B) SDS–PAGE with the fusion proteins that had been purified using Ni^{+}–NTA agarose resin.

EGFP-PTD(1), -PTD(2), -PTD(3), -PTD(4), and -PTD(5), as well as the pET-32a(+) empty vector. Each protein was expressed in the presence of 1 mM IPTG in the bacteria. When the proteins were analyzed in SDS–PAGE gel, the overexpressed fusion proteins were obvious in the bacterial lysates that have been treated with IPTG (Fig. 2A). Before applying to the cells, the fusion proteins were partially purified from the bacterial lysates using Ni^{+}–NTA agarose resin followed by dialysis against PBS. The purified fusion proteins were analyzed in SDS–PAGE gel, and the expected size of each protein was confirmed: no insertion, 20.4 kDa; EGFP only, 48.8 kDa; EGFP-PTD(1), 51.1 kDa; EGFP-PTD(2), 53.5 kDa; EGFP-PTD(3), 55.9 kDa; EGFP-PTD(4), 58.2 kDa; EGFP-PTD(5), 60.6 kDa (Fig. 2B). The purification yield of the EGFP-PTD(1) was about 6.6% of total proteins (Table 1), and comparable yields were obtained from other fusion proteins (data not shown).

Table 1
Purification of recombinant Hox -PTD (1) from *E.coli*

Purification step	Total protein (mg)[a]	Yield (%)
Crude extracts[b]	245	100
Ni^{+2} chelating column	16.22	6.6

[a] Total protein concentration was determined by Bradford protein assay, using bovine serum albumin (BSA) as a standard.

[b] The starting material was crude extracts from the lysis of 700 ml bacterial culture pellet.

Transduction efficiencies of the EGFP-PTD fusion proteins

To test whether the 16 amino acid peptide can induce the internalization of the fusion proteins, the purified EGFP-PTD(1) proteins were applied to the cultures of the primary pig fetal fibroblast (PPFF) cells at different concentrations ranging from 50 nM to 2 μM, and incubated for 48 h. After washing off unbound proteins,

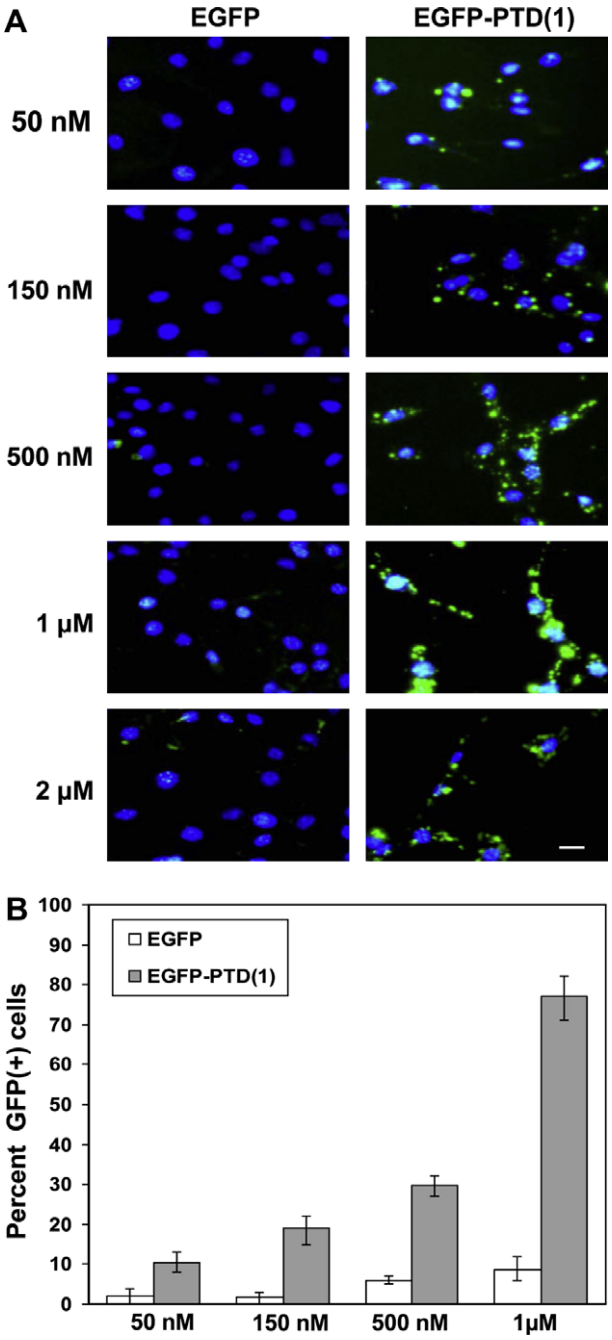

Fig. 3. Membrane transducing activity of the EGFP-PTD(1) fusion protein is dose-dependent. (A) 50 nM, 150 nM, 500 nM, 1 μM, or 2 μM of EGFP-PTD(1) proteins were applied to the cultures of primary pig fetal fibroblast cells. The fusion proteins were efficiently internalized into the primary cells, whereas EGFP alone was not. Scale bar represents 25 μm. (B) GFP-positive cells were quantified by FACS analysis. Percentages of GFP-positive cells were increased with higher concentrations of EGFP-PTD(1) applied. Nearly 80% of cells were GFP-positive with 1 μM EGFP-PTD(1), whereas less than 10% of cells showed GFP-fluorescence with EGFP alone.

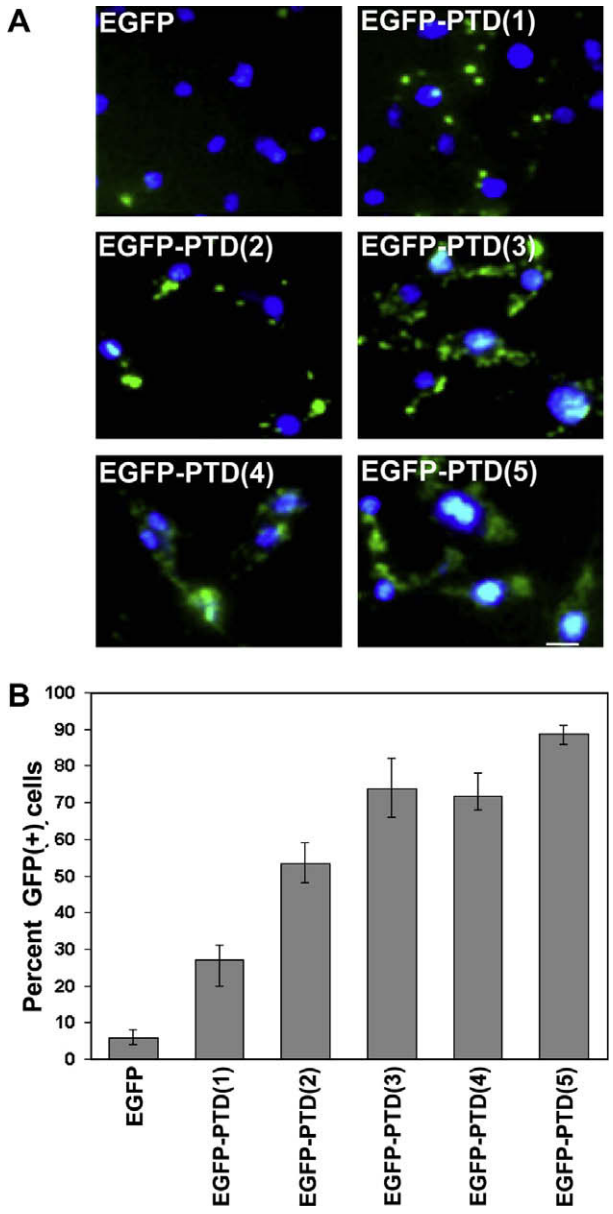

Fig. 4. Membrane transducing activities of the fusion proteins are dependent on the number of PTD copies. (A) PPFF cells were applied with 500 nM of EGFP, EGFP-PTD(1), EGFP-PTD(2), EGFP-PTD(3), EGFP-PTD(4) and EGFP-PTD(5) for 24 h, and then the EGFP signals were analyzed under a fluorescence microscope. Scale bar represents 25 μm. (B) FACS analysis of GFP-positive cells. Percentages of GFP-positive cells were increased with higher copy numbers of PTD fused to EGFP.

green fluorescence of the EGFP-PTD(1) was observed under a fluorescent microscope. Green fluorescence of the internalized EGFP-PTD(1) proteins was detected even at the lowest concentration applied (50 nM), and the transduction efficiency was increased in a concentration-dependent manner up to 1 μM, where almost 80% of cells were GFP-positive (Fig. 3A and B). There was no further increase in the transduction efficiency above 1 μM. In contrast to the EGFP-PTD(1), EGFP alone did not seem to traverse the membrane (Fig. 3A and B), although weak signals were detected in higher concentrations, probably due to non-specific protein binding onto the PPFF cells which were not washed off completely during the washing step.

After confirming the ability of the 16 amino acid peptide, which is only 2.23 kDa, to deliver 49 kDa fused proteins into the cells, we next examined the transduction efficiencies of the fusion proteins that contain multiple copies of PTDs; EGFP-PTD(1), EGFP-PTD(2), EGFP-PTD(3), EGFP-PTD(4), and EGFP-PTD(5). Each fusion protein was added to the PPFF cells at a concentration of 500 nM and cultured for 24 h. Interestingly, the number of GFP-positive cells increased up to PTD(3) and then seemed to plateau (Fig. 4), indicating that the transduction efficiency of the fusion proteins is dependent on the number of PTD copies attached .

Our results clearly demonstrate that the 16 amino acid third helix in the homeodomain of murine Hoxc8 is sufficient to facilitate the transduction of fusion proteins into the mammalian cells, acting as an efficient PTD. The efficiency of the transduction induced by the Hoxc8 PTD was enhanced when higher concentrations of the EGFP-PTD fusion proteins were applied to the cells, or more copies of the PTD peptides were fused to EGFP, indicating that the transduction activity of the Hoxc8 PTD is dose-dependent. Similar transduction results were obtained in experiments with F9 (murine teratocarcinoma) and COS-7 (monkey kidney fibroblast) cells, although the transduction efficiencies were lower than that with PPFF cells (data not shown). Based on our previous study showing the ability of Hoxc8 protein to form a complex with DNA molecules and deliver them into the cells [16], we are currently testing if the 16 amino acid Hoxc8 PTD can also serve as a delivery vehicle for macromolecules such as expression vectors.

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