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Enhanced in-cell folding of reversibly cationized transcription factor using amphipathic peptide

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The intracellular delivery of functionally active transcription factor proteins is emerging as a promising technique for artificial regulation of cellular functions. However, in addition to the cell membrane, which acts as a barrier to macromolecules, the aggregation-favored properties of structurally flexible transcription factor proteins limit the application of this method. In-cell folding technique can be used to overcome these issues. This technique solubilizes denatured protein by reversible alkyl-disulfide cationization (*S*-cationization), and simultaneously endows efficient intracellular delivery and folding to the biologically active conformation in the reducing environment of the cytosol. Because cationized protein is internalized into cells by adsorption-mediated endocytosis, endosomal escape is crucial for this technique. In this study, we utilized a sensitive luciferase reporter gene assay to quantitatively evaluate in-cell folding of the artificial transcription factor GAL4-VP16. Although the cationic moiety of *S*-cationized protein was slightly affected, co-transduction of amphipathic peptide Endo-PORTER dramatically improved in-cell folding efficiency. Live cell imaging of fluorescent-labeled GAL4-VP16 revealed that some of the proteins diffused into the cytosol and nucleus through co-transduction with Endo-PORTER. Real-time monitoring of light output of luciferase revealed the kinetics of in-cell folding, supporting that endosomal-release assisted by Endo-PORTER was stimulated by endosome acidification. Because this method can transduce proteins uniformly and repeatedly into living cells, *S*-cationized transcription factor proteins are widely applicable for the artificial regulation of cellular functions.

[**Key words:** Protein transduction; Cell-permeable peptide; In-cell folding; Amphipathic peptide; Chemical modification]

Somatic cells have specialized functions in different tissues and organs. Perfectly designed cellular gene expression machinery is regulated by nuclear transcription factors. Therefore, specific transcription factors can be used to artificially regulate cellular functions. Transient transfection of plasmid DNA or mRNA encoding transcription factors and direct transcription factor protein transduction into living cells are alternative methods for expressing protein functions. These methods require efficient macromolecule intracellular delivery through the cell membrane barrier without affecting physiological cellular functions.

We previously developed efficient protein transduction techniques based on chemical protein cationization (1–6). Cationized proteins show rapid and uniform adsorption onto the cellular surface and internalization into cells via the endocytosis-like pathway (2,3,7). Although highly cationized proteins with multiple amidation of carboxyl groups by diamine (1,7,8) or cationic polymer (2–4,6,9–14) show efficient intracellular delivery, protein cationization techniques must be optimized for each protein property. Additionally, direct protein chemical cationization can only be used for robust proteins (1,2,6,8,12,13). In order to minimize adverse effects on effector proteins, the cationic carrier system was developed based on the intermolecular interaction of biotin/avidin (3,6,10,14), immunoglobulin/Protein-G (3), or glutathione/glutathione *S*-transferase (9). However, transcription factor transduction requires more sophisticated cationization methods to maintain proper function in cells (6). Transcription factors must bind to specific recognition sites on the chromosome and interact with other proteins in the nucleus. The cationic moiety for protein transduction can interfere with molecular recognitions. Because recombinant transcription factors are often produced as insoluble inclusion bodies in *Escherichia coli*, protein solubilization is required before transduction. The in-cell folding technique can be used to overcome above issues, which solubilize insoluble protein by reversible alkyl-disulfide cationization, simultaneously endowing intracellular delivery and folding to the biologically active conformation in the reducing cytosolic environments (4,5).

Cationic protein transduction is thought to occur mainly through internalization into cells via endocytosis-like pathways after electrostatic adsorption onto the cellular surface (2,3,5). Because internalized proteins encapsulated in endosome-like vesicles are no longer functional, the rate of release from the endosomal vesicle into the cytosol determines the actual protein

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transduction efficiency (7,15). Several strategies have been used to enhance the endosomal escape of internalized protein by co-transduction with amphipathic peptides (16–19) or photosensitizers (20,21). Weak-base amphipathic peptide Endo-PORTER composed of Leu and His rapidly adsorb to cellular surface and is internalized by endocytosis. Following acidification of endosome, amphipathicity of Endo-PORTER is enhanced, resulted in destabilization of endosomal vesicles (22). Endo-PORTER mainly employed for transduction of morpholino oligonucleotides or siRNAs, as well as available for protein transduction (7,14,23–26). Using this strategy, we have successfully enhanced cytosolic protein transduction of cationized RNase (7) and cationized avidin carrier (14). Non-treated proteins also demonstrated cytoplasmic protein delivery via endocytosis with Endo-PORTER, for example, enhanced cross presentation of antigen uptake on dendritic cells (23) or functional protein transduction to macrophage (26). In this study, we optimized in-cell folding of transcription factors by enhancing endosomal escape. This method for sophisticated artificial regulation of specific gene expression by in-cell folding of transcription factor can be used to manipulate cellular functions.

MATERIALS AND METHODS

Protein expression and purification The primary structure of GAL4-VP16 artificial transcription factor (27) is presented in Fig. 1A. The DNA binding domain (N-terminal 147 amino acids) of the yeast transcriptional activator GAL4 was genetically fused with the C-terminus of the activation domain of VP16 protein herpes simplex virus (C-terminal 130 amino acids) through a linker sequence with a His-Tag (GGSHHHHHHGGS) to prepare GAL4-VP16. cDNA encoding GAL4-VP16 was cloned as an *Nde*I/*Not*I fragment into the polylinker of pET-21b (Novagen, Madison, WI, USA). The expression and purification of fusion protein were performed as described previously (27). Briefly, His-Tagged GAL4-VP16 protein was expressed in *E. coli* BL21-CodonPlus(DE3)-RIPL (Stratagene, La Jolla, CA, USA) in Terrific Broth containing 20 μM of $ZnSO_4$. The cells were resuspended in the lysis buffer consisting of 20 mM HEPES-KOH, pH 7.5, 20% glycerol, 100 mM NaCl, 10 μM $ZnSO_4$, 1 mM PMSF, and 20 mM imidazole, and were disrupted by sonication on ice. Recombinant protein was purified using TALON Metal Affinity Resin (Takara Bio, Shiga, Japan).

Chemical modification Cysteine sulfhydryl group cationization (*S*-cationization) (28) was employed to solubilize denatured protein. Affinity-purified GAL4-VP16 protein, dissolved in lysis buffer containing 200 mM imidazole, was denatured by adding solid guanidine hydrochloride (GdnHCl) at a final concentration of 6 M and reduced with 30 mM of dithiothreitol at 37°C for 1 h. The denatured and reduced protein was cationized by addition of TAPS-sulfonate (Katayama Chemical, Osaka, Japan), APS-sulfonate (4) or (3-bromopropyl)trimethylammonium bromide (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 70 mM. After incubation at 37°C for 30 min, TAPS-, APS-, or TAP-modified GAL4-VP16 protein (TAPS-GAL4-VP16, APS-GAL4-VP16, or TAP-GAL4-VP16) solution was acidified by addition 1/10 volume of acetic acid and dialyzed against 0.1% acetic acid. The *S*-cationization with polyethylenimine (averaged molecular mass 300, PEI300, Nippon Shokubai, Tokyo, Japan) of denatured and reduced protein has been described previously (4).

N-terminal specific fluorescence labeling of TAPS-GAL4-VP16 protein was performed in a transamination reaction using pyridoxal-5-phosphate and Hilyte Fluor 488 hydrazide (AnaSpac, Fremont, CA, USA) (29). Briefly, GAL4-VP16 protein dissolved in 6 M GdnHCl containing 50 mM sodium phosphate, pH 6.5, and 10 mM ethylenediaminetetraacetic acid (EDTA) was treated with 10 mM of pyridoxal-5-phosphate at 37°C for 24 h. The resultant N-terminus keto-protein product was recovered as a precipitate after dialysis against water. After dissolving the precipitates in 6 M GdnHCl containing 50 mM sodium phosphate, pH 6.5, and 10 mM EDTA, the N-terminus keto-protein product was treated with 1 mg/mL of Hilyte Fluor 488 hydrazide at 37°C for 72 h. After dialysis against water, N-terminus specific fluorescence-labeled GAL4-VP16 was recovered as a precipitate. This product was solubilized by *S*-cationization with TAPS-sulfonate as descried above.

Cell culture and protein transduction The HeLa luciferase receptor (HLR) cell line, which contains a GAL4-binding element-TATA sequence followed by the gene encoding firefly luciferase, was purchased from Stratagene. HLR cells were maintained according to the manufacturer's instructions. Endosome destabilizing peptide Endo-PORTER (Gene Tools, Philomath, OR, USA) was dissolved in dimethyl sulfoxide at a concentration of 1 mM. HLR cells were seeded into a 6-well plate or

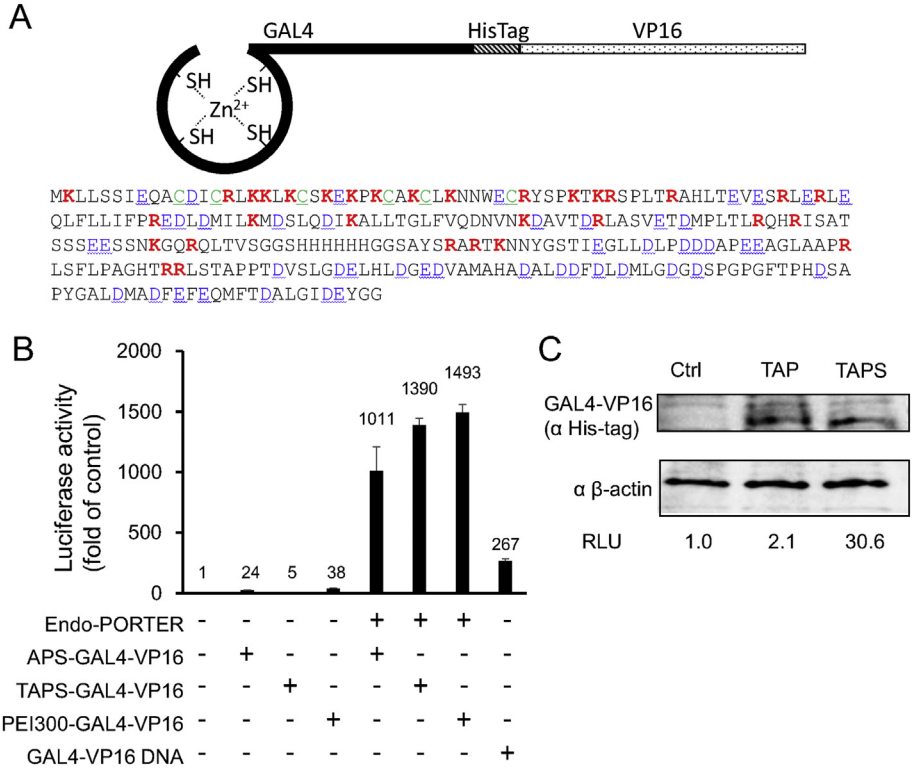

FIG. 1. (A) Schematic drawing and primary structure of GAL4-VP16 fusion protein. N-terminal domain of GAL4 possesses zinc finger like motif. Positively and negatively charged residues and cysteines are respectively shown in bold (red), double wavy underline (blue), and underlined (green). (B) Luciferase activity of HLR cells treated with various *S*-cationized GAL4-VP16 derivatives with or without Endo-PORTER. (C) Both irreversibly *S*-cationized TAP-GAL4-VP16 and reversibly *S*-cationized TAPS-GAL4-VP16 were confirmed to be equivalently internalized into HLR cells by western blotting, but showed impaired in-cell folding. RLU means relative luciferase activity determined by using each cell lysate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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