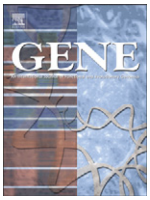
Gene 489 (2011) 89–97

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene



Gene transport and expression by arginine-rich cell-penetrating peptides in *Paramecium*

Yun-Hao Dai [a], Betty Revon Liu [a,b], Huey-Jenn Chiang [b], Han-Jung Lee [a,*]

[a] *Department of Natural Resources and Environmental Studies, National Dong Hwa University, Hualien 97401, Taiwan*
[b] *Graduate Institute of Biotechnology, National Dong Hwa University, Hualien 97401, Taiwan*

ARTICLE INFO

Article history:
Accepted 25 August 2011
Available online 7 September 2011

Received by A.J. van Wijnen

Keywords:
Cell-penetrating peptide (CPP)
Green fluorescent protein (GFP)
3-(4,5-Dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide (MTT) assay
Paramecium caudatum
Transformation
Transgenesis

ABSTRACT

Owing to the cell membrane barriers, most macromolecules and hydrophilic molecules could not freely enter into living cells. However, cell-penetrating peptides (CPPs) have been discovered that can translocate themselves and associate cargoes into the cytoplasm. In this study, we demonstrate that three arginine-rich CPPs (SR9, HR9 and PR9) can form stable complexes with plasmid DNA at the optimized nitrogen/phosphate ratio of 3 and deliver plasmid DNA into *Paramecium caudatum* in a noncovalent manner. Accordingly, the transported plasmid encoding the green fluorescent protein (*GFP*) gene could be expressed in cells functionally assayed at both the protein and DNA levels. The efficiency of gene delivery varied among these CPPs in the order of HR9 > PR9 > SR9. In addition, these CPPs and CPP/DNA complexes were not cytotoxic in *Paramecium* detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide (MTT) assay. Thus, these results suggest that the functionality of arginine-rich CPPs offers an efficient and safe tool for transgenesis in eukaryotic protozoans.

1. Introduction

Paramecium is a ciliated unicellular model organism for many aspects of cell biology (Vincensini et al., 2011). Its size of about 100 μm allows for easy manipulation and observation (Hauser et al., 2000). This eukaryotic protozoan contains both a somatic macronucleus and a germinal micronucleus within the unicell (Takenaka et al., 2007). The macronucleus is highly polygenomic and generated from a micronucleus following either conjugation or autogamy (Takenaka et al., 2001). *Paramecium* has periods of sexual immaturity, maturity, senescence and finally cell death. Replacement of an old macronucleus with a newly processed macronucleus from the micronucleus by conjugation or autogamy is necessary to restart the clonal life cycle (Takenaka et al., 2001).

It has been documented that paramecia can be transfected (transformed) by microinjection (Knowles, 1974), electroporation (Boileau et al., 1999; Fraga et al., 1998), particle bombardment (Boileau et al., 1999; Fraga et al., 2006) and bacterial infection (Sabaneyeva et al., 2009). The green fluorescent protein (*GFP*) reporter gene was an alternative to replace the traditional antibiotic screening method for selecting transformed cells (Hauser et al., 2000). Recently, several *GFP* coding genes varying in codon usage have become a popular selecting marker for transformed paramecia (Hauser et al., 2000; Sabaneyeva et al., 2009; Takenaka et al., 2002, 2007).

The plasma membrane of eukaryotic cells/organisms is an effective barrier to most macromolecules (such as proteins and nucleic acids) and hydrophilic molecules. The ability to efficiently and safely transfect exogenous genes into a host genome, such as *paramecium*, is essential for studies of cellular processes, functional genomics and transgenesis. Currently, the most commonly used vectors for transient or permanent transfer of genes are virus-based carriers (Urshitz et al., 2010). Viral carriers usually have high transfection efficiency, but are limited in some factors, such as cargo size, production costs and biosafety considerations. To avoid some drawbacks caused by viral systems, nonviral carriers are also being developed extensively as an alternative in a diverse field of applications.

Cell-penetrating peptides (CPPs) have recently drawn enormous attention as a powerful nonviral tool for the direct intracellular delivery of proteins, genes, siRNA and nanoparticles (Gump and Dowdy, 2007). In 1988, transactivator of transcription (TAT) protein from the HIV type 1 was discovered to pass through the plasma membrane and arrive at nucleus (Frankel and Pabo, 1988; Green and Loewenstein, 1988). After the study of TAT, 11 amino acids (YGRKKRRQRRR) were found to be the functional domain which contributes to the ability of translocation into cells and brings cargos into cells (Vives et al., 1997). Subsequently, many peptides possessing the cellular uptake

---

Abbreviations: CPP, cell-penetrating peptide; Cy3, Cyanine 3; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide; N/P, nitrogen (NH_3^+)/phosphate (PO_4^-); PKI, protein kinase inhibitor; PTD, protein transduction domain; RBC, red blood cell; RFP, red fluorescent protein; SRB, sulforhodamine B; TAT, transactivator of transcription; Tp2, T cell antigen of *Theileria parva*; ZFN, zinc finger nuclease.

[*] Corresponding author. Tel.: +886 3 8633642; fax: +886 3 8633260.
E-mail address: hjlee@mail.ndhu.edu.tw (H.-J. Lee).

0378-1119/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2011.08.011

activity were called cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) (Deshayes et al., 2010). CPPs have some common characteristics which mainly consist of basic amino acids, such as arginine and lysine. Among them, polyarginine tended to have higher ability of translocation efficiency than others (Futaki, 2002). Therefore, CPPs became an attractive approach in opening new perspectives for gene delivery during the last decade (Deshayes et al., 2010).

In recent years, we have demonstrated that a CPP (synthetic nona-arginine; SR9) is able to transport fluorescent protein cargos in a non-covalent manner into living animal and plant cells (Chang et al., 2007; Hou et al., 2007; Hu et al., 2009; Lu et al., 2010; Wang et al., 2006) and into other organisms including cyanobacteria, bacteria, archaea and yeasts (Liu et al., 2008). Moreover, this SR9 could deliver DNA (Chen et al., 2007; Lee et al., 2011), RNA (Wang et al., 2007) or nanoparticles (Liu et al., 2010a,b, 2011; Xu et al., 2010) into living cells noncovalently. Recently, three arginine-rich CPPs (SR9, HR9 and PR9) were investigated in our group (Liu et al., 2011). However, limited study of CPPs was presented in the field of eukaryotic protista (Nain et al., 2010; Ryves and Harwood, 2006; Tineg et al., 2009).

The aim of this work was for the first time to investigate the ability of three arginine-rich CPPs to be internalized, *in vitro* interact with DNA and deliver DNA into paramecia. Intracellular DNA transport mediated by CPPs could be determined by gel retardation assay, trafficking of *in vitro* fluorescent labeled DNA and the functional gene assay by the gene expression of the *GFP* reporter gene-containing plasmid at the protein and DNA levels. Finally, any cytotoxicity caused by DNA, CPPs or CPP/DNA complexes in paramecia was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide (MTT) assay.

2. Materials and methods

2.1. Culture of *Paramecium caudatum*

Paramecia (*P. caudatum*) were grown in the culture medium of 1.25% (w/v) fresh lettuce juice (Takenaka et al., 2002) diluted with the Dryl's solution (Strahl and Blackburn, 1994). Young cells with 4–5 divisions were maintained at 23–25 °C and seeded at a density of 50–100 paramecia per 100 μl in a well of 24-well plates for 1 day at room temperature before experiment.

2.2. Preparation of peptide, protein and plasmid

Three arginine-rich CPPs (SR9, HR9 and PR9) were synthesized as previously described (Liu et al., 2011). The M_r of SR9 (R9), HR9 (C-5H-R9-5H-C) and PR9 (FFLIPKG-R9) peptides was 1423.7, 3001.7 and 2328.9 Da, respectively. HR9-FITC (FITC-C-5H-R9-5H-C) peptide of 88.2% purity was synthesized to contain the fluorescein isothiocyanate (FITC) at the N-terminus (Genomics, Taipei, Taiwan). Alexa Fluor 488-5′-dUTP (Invitrogen, Carlsbad, CA, USA) was used as a fluorescent dye. Insulin-FITC (Sigma-Aldrich, St. Louis, MO, USA) was applied as a non-CPP reference as previously described (Hou et al., 2007). GFP was purified from *Escherichia coli* transformed with the pGFP1 plasmid (Lu et al., 2010) as previously described (Chang et al., 2005a).

The pGFP-actin1-1 plasmid (kindly provided by Dr. Ilya N. Skovorodkin, University of Oulu, Finland) containing 5828 bp was described previously (Sabaneyeva et al., 2009). The coding sequence of the GFP-actin fusion construct is under the control of the *P. caudatum* α-tubulin promoter.

2.3. CPP entry into paramecia

To observe cellular uptake of CPP, young paramecia were washed in water before treatment. Cells were treated with 7.15 μM of HR9-FITC for 1 h at room temperature. The culture plate was then drained and washed with water carefully. The cells were subsequently monitored right after incubation with 1% formaldehyde fixation using the BD Pathway 435 system (BD Biosciences, Franklin Lakes, NJ, USA).

2.4. Gel retardation assay

To reveal any interactions between DNA and CPP, gel retardation assay was carried out as previously described (Chen et al., 2007). The proportion between amino nitrogen (NH_3^+) of peptide and phosphate (PO_4^-) of DNA was defined as the N/P ratio (Chen et al., 2007). Briefly, 1 μg of the pGFP-actin1-1 plasmid (17.3 nM) was mixed with CPPs (PR9: 7.1, 14.3, 28.5, 42.8, 57.0, 85.5, 114.0, 142.6, 171.1 and 199.6 μM or HR9: 5.3, 10.6, 21.2, 31.7, 42.3, 63.5, 84.6, 105.8, 126.9 and 148.1 μM) to form different N/P ratios (0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 14) in double-deionized water at a final volume of 15 μl and incubated at 37 °C for 1 h. These mixtures were analyzed by electrophoresis on a 1% agarose gel at 50 Vol for 30 min and stained by EtdBr. Images were captured using the Typhoon FLA 9000 biomolecular imager (GE Healthcare, Piscataway, NJ, USA) as previously described (Liu et al., 2011).

2.5. In vitro DNA labeling

To prepare *in vitro* labeled DNA, the pBlueScript-SK+ plasmid DNA (Agilent Technologies, Santa Clara, CA, USA) was labeled with the *Label*IT Cyanine 3 (Cy3) nucleic acid labeling kit according to the manufacturer's instructions (Mirus Bio, Madison, WI, USA) as previously described (Chen et al., 2007).

2.6. CPP-mediated DNA delivery into paramecia

DNA delivery into paramecia was mediated by CPPs as previously described (Chen et al., 2007). Three μg of the Cy3-labeled pBlueScript-SK+ plasmid DNA was mixed without or with CPPs (SR9, HR9 or PR9) at the N/P ratio of 3 in water at a final volume of 60 μl and incubated for 1 h at room temperature. Cy3-labeled DNA only or CPP/Cy3-labeled DNA complexes were then dropped into cells and incubated for 30 min at room temperature. After the incubation, the cells were added with 180 μl of the Dryl's solution and monitored without fixation by a fluorescent microscope.

For the functional gene assay, 3 μg of the circular pGFP-actin1-1 plasmid without telomeres was mixed without or with CPPs (SR9, HR9 or PR9) at the N/P ratio of 3 in water at a final volume of 60 μl and incubated for 1 h at room temperature. The pGFP-actin1-1 plasmid only, CPPs only or CPP/pGFP-actin1-1 complexes were then dropped into cells and incubated for 30 min at room temperature. After the incubation, the cells were added with 1 ml of culture medium and incubated for additional 3 days at room temperature. Finally, the cells were monitored with fixation using the BD Pathway 435 system (BD Biosciences).

2.7. PCR

Paramecia were treated without or with the pGFP-actin1-1 plasmid according to the functional gene assay. After the treatment, all the cells were treated with DNase (10 unit/μl, Promega, Madison, WI, USA) for 30 min at 37 °C to remove extracellular DNA and heat-inactivated for 15 min at 80 °C. The pGFP-actin1-1 plasmid as a positive control and lysates of the cells with various treatments were PCR-amplified using primers GFP5 (5′-AAGAATTCCATGGTGAGCAAGGGCGAGGAGCTGTT-3′) and GFP6 (5′-AAGAATTCTTAAGCTTGTACAGCTCGTCCATGCCGA-GAGT-3′) as previously described (Li et al., 2010). Images were captured using the Typhoon FLA 9000 biomolecular imager.