Algal Research 24 (2017) 360–367

Contents lists available at ScienceDirect

Algal Research

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

A highly efficient cell penetrating peptide pVEC-mediated protein delivery system into microalgae

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ARTICLE INFO

Keywords:
Microalgae
Cell-penetrating peptides (CPPs)
Protein delivery
Biomass

ABSTRACT

Interest in microalgae has significantly increased due to its potential as a promising bioresource in various fields. However, a technique for intracellular delivery of proteins into microalgae has not established yet, although it is potentially valuable for advanced algal research. Here, we propose a cell penetrating peptide pVEC-mediated protein delivery tool for microalgae. We discovered that the peptide pVEC from vascular endothelial cadherin was more effective in transporting exogenous proteins into algal cells than other peptides including R9, Transportan, TAT and Penetratin. pVEC-mediated tool was shown to deliver proteins of various sizes from 6 kDa to 150 kDa into wild-type *Chlamydomonas reinhardtii* and to also work in other algal species such as *Nannochloropsis salina* and *Chlorella vulgaris* when delivering a 66 kDa protein. In addition, we show that our proposed pVEC-mediated protein delivery system is based on both the endocytic and non-endocytic pathways simultaneously through a mechanism study using inhibitors such as low temperature, *N*-ethylmaleimide, 5-(*N*-ethyl-*N*-isopropyl) amiloride, chlorpromazine, sodium azide, and methyl-beta-cyclodextrin. The results show that the proposed simple and efficient protein delivery tool could contribute to advanced algal research for a wide array of applications for microalgae in various industries.

1. Introduction

In the last few decades, microalgae, eukaryotic photosynthetic microorganisms that can grow at a rapid rate and live in harsh conditions [1], have been considered as a promising bioresource. Microalgae accumulate diverse nutraceuticals and pharmaceutical compounds in their cell bodies that can be used in the food and pharmaceutical industry [1–3]. Some research has shown that microalgae not only can generate valuable products but also can effectively fix carbon dioxide which could be part of the solution for the carbon dioxide gas emission issue [4–6]. Recently, microalgae have received significant attention as a bioresource to produce biodiesel because they accumulate lipids in their cell bodies ranging between 20% to 50% of their dry cell weight under certain conditions [1,7,8]. Many studies have shown that the conventional biofuel production system based on the agricultural biomass, which has severe limitations, could be replaced with an advanced biodiesel production system that uses specific algal species [8,9]. However, for the practical use of microalgae in biodiesel production, improving their biological characteristics has been considered essential, especially the photosynthetic efficiency, growth rate, biomass production rate and oil contents in the cell body. For that reason, it has been a major focus of many basic biological science or genetic engineering studies [10,11].

Intracellular delivery of proteins is an important technique in biological science. Delivery of proteins into cells enables the investigation of protein-protein interactions and their functions in a cell system [12–16]. Considering that most biological reactions are protein based reactions, the intracellular delivery of proteins is an excellent approach for fundamental biological research which is the groundwork for future applications. Recently, the delivery of proteins has become a hot issue after the development of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system which enables genome editing with high target specificity and simplicity [17,18]. While numerous studies have covered the delivery of molecules such as proteins into mammalian cells, only a few studies have investigated the intracellular delivery of molecules into microalgae due to the difficulties of passing through the algal cell wall and the membrane barrier and the relatively limited amount of research compared to mammalian cell lines [19,20].

Here, we investigated the highly efficient delivery of proteins into microalgae using the cell penetrating peptide pVEC, an amphipathic 18-amino acid-long peptide derived from vascular endothelial cadherin, in

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http://dx.doi.org/10.1016/j.algal.2017.04.022
Received 14 December 2016; Received in revised form 23 March 2017; Accepted 21 April 2017

its non-covalent form. In our previous study investigating the penetration of cell penetrating peptides conjugated with fluorescein isothiocyanate (FITC), we reported that pVEC is the most effective cell penetrating peptide among the various cell penetrating peptides for *Chlamydomonas reinhardtii* [21]. Without any effort to covalently link target proteins to the cell penetrating peptide by chemical method, proteins of various sizes from 6 kDa to 150 kDa were effectively delivered into wild-type *Chlamydomonas reinhardtii* (*CC-124*) with pVEC in this study. In addition to *Chlamydomonas reinhardtii*, pVEC effectively delivered proteins into the *Chlorella vulgaris* and *Nannochloropsis salina* which are regarded as promising species for biofuel production. Furthermore, we investigated whether the endocytic pathway or non-endocytic pathway (direct pathway) are involved in the delivery of the proteins in a mechanism study using several endocytosis inhibitors. This study will greatly contribute to the basic biology of algae as a new molecular manipulation tool. Additionally, our system will be used in genetic engineering associated with the CRISPR/Cas9 system as an efficient cas9 delivery tool in the future.

2. Materials and methods

2.1. Microalgae growth condition and preparation

Microalgae *Chlamydomonas reinhardtii* (CC-124) and *Chlorella vulgaris* were cultivated in tris-acetate phosphate (TAP) medium under continuous illumination, constant agitation (about 140 rpm), and constant temperature (25 °C). *Nannochloropsis salina* was cultivated in a modified F2N media consisting of 15 g/L sea salt (Sigma-Aldrich, USA), 10 mM Tris–HCl (pH 7.6), 427.5 mg/L $NaNO_3$, 5 mL/L trace metal mixture (4.36 g/L Na_2 EDTA·$2H_2O$, 3.15 g/L $FeCl_3$·$6H_2O$, 10 mg/L $CoCl_2$·$6H_2O$, 22 mg/L ZnSO4·$7H_2O$, 180 mg/L $MnCl_2$·$4H_2O$, 9.8 mg/L CuSO4·$5H_2O$, and 6.3 mg/L Na_2MoO_4·$2H_2O$), 30 mg/L NaH_2PO_4·$2H_2O$, and 2.5 mL/L vitamin stock (1 mg/L vitamin B_{12}, 1 mg/L Biotin, and 200 mg/L thiamine·HCl). The cultivation condition was the same as that for *Chlamydomonas* and *Chlorella* except for the supply of air with 2% CO_2 to the broth at 0.5 vvm (column gas per volume per minute) [22].

At the exponential phase of all cell lines, the cells were harvested and resuspended in new media at a final concentration of 1.4×10^8 cells/mL with a hemocytometer.

2.2. Intracellular delivery of proteins with the cell penetrating peptide

The cell penetrating peptides (CPPs) used in this study including R9 (RRRRRRRRR, 1424 Da), pVEC (LLIILRRRIRKQAHAHSK, 2209 Da, C-terminal amidated), Transportan (GWTLNSAGYLLGKINLKALAALAKKIL, 2841 Da), TAT (YGRKKRRQRRR, 1622 Da), and Penetratin (PEN, RQIKWFQNRRMKWKK, 2246 Da) were purchased from Peptron (Daejeon, Korea). The solutions of CPPs with specific concentrations were added to cell samples at the same volume ratio resulting in a final concentration of 7×10^7 cells/mL. The target proteins conjugated with FITC were added to the cell-CPP samples at a final concentration of 10 μM or 20 μM. After a 15 min reaction time at 25 °C, the cells were washed with TAP media using a centrifuge, and the supernatants were discarded. After the washing, the samples were treated with trypsin for 15 min at 37 °C to effectively eliminate any fluorescent protein bound to the cell surface because it was observed that FITC-proteins not delivered into the cell strongly bind to the cell surface in the absence of trypsin treatment (Supplementary Fig. 1). Although the reason for this phenomenon is not clear, CPPs (especially pVEC) seem to promote the adhesion of proteins to the surface of cells. After trypsin treatment, the cells were washed two times with media with a centrifuge.

2.3. Measurement of molecular uptake efficiency and viability

In this study, three kinds of FITC conjugated proteins, insulin, bovine serum albumin (BSA) and alcohol dehydrogenase were used whose molecular weights are 6, 66, and 150 kDa, respectively. FITC-insulin was prepared with a standard FITC conjugation method described in a previous study. FITC-BSA and alcohol dehydrogenase were purchased from Sigma Aldrich. The FITC conjugation to alcohol dehydrogenase was done by a professional company (BioActs, Korea).

The intracellular molecular uptake efficiency was measured with a cell cytometer (FACS) (MoFlo XDP, Beckman Coulter, USA). The cell was regarded as “fluorescent” if its fluorescence intensity was greater than the background signal from 99% of the untreated control cell. The efficiency was calculated by dividing the number of fluorescent cells by the number of total cells. The fluorescence intensity of the bulk sample was measured by a spectrofluorophotometer (RF-5301pc, Shimadzu, Japan) at 511 nm with an excitation wavelength of 475 nm. The image for cellular uptake was took by confocal laser scanning microscopy (FV1000 Live, Olympus, Japan).

The viability after treating the cells with the CPPs was assayed by measuring the quantum yield (QY) of chlorophyll in microalgae and the number of colonies indicating the number of cells recovered. QY was measured with the AquaPen-C APA100 (Czech Republic). The samples were incubated for one hour in the dark for dark adaptation. The ratio of the quantum yield was calculated by dividing the QY of the experimental group by the QY of the control group. For the number of colonies, algal cells treated with CPPs were transferred to 10 mL of TAP media for 8 h at 25 °C under dim light. After a series of dilutions, 400 cells were spread onto agar plates comprised of TAP media with 1.5% agar powder. The agar plates were incubated at 25 °C under continuous illumination for about 10 days.

2.4. Mechanistic study of the cellular uptake using inhibitors

As mentioned in previous papers [23,24], physical and pharmacological inhibitors were used including low temperature (4 °C), chlorpromazine (CPZ), sodium azide (SA), 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), *N*-ethylmaleimide (NEM) and methyl-beta-cyclodextrin (MβCD), respectively. All the inhibitors were purchased from Sigma Aldrich. The cells were treated with each inhibitor for 1 h. The final concentrations of CPZ, SA, EIPA, NEM and MβCD were 1.5 mM, 10 mM, 100 μM, 2 mM and 150 mM, respectively. After 1 h, the inhibitors were washed out by centrifugation at 5000 rpm, and the cells were resuspended in media at a final concentration of 7×10^7 cells/mL. The CPP and FITC-BSA were added to cells treated with the inhibitors. After treatment for 15 min and a series of washing steps mentioned above (Section 2.2), the fluorescent intensity of the sample was measured in the same manner as in the previous section.

2.5. Statistical analysis

At least three replicates (n = 3) were used to measure the delivery efficiency, viability, and fluorescent intensity. Error bars whose values were derived from dividing each standard deviation by the square root of 3 are shown in figures. Significant differences between sample means were analyzed with Student's *t*-test following significant analysis of variance results at $p < 0.05$(*).

3. Results and discussion

3.1. Screening of cell penetrating peptides for the delivery of proteins

Previously, there have been only a few studies regarding the application of CPPs in microalgae such as the translocation of cell penetrating peptides (CPPs) labeled with FITC [19,21,25], delivery of small dsRNA fragments by a CPP [25] and delivery of proteins with a covalently linked form [19]. Most of these studies used the arginine (R)-rich cell penetrating peptide (R7 ~ R9) for the delivery of molecules which is one of the most well-known cell penetrating peptides. In a