Expressed protein ligation for the preparation of fusion proteins with cell penetrating peptides for endotoxin removal and intracellular delivery

Hao-Hsin Yu, Ikuhiko Nakase, Silvia Pujals, Hisaaki Hirose, Gen Tanaka, Sayaka Katayama, Miki Imanishi, Shiroh Futaki

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

A R T I C L E   I N F O

Article history:
Received 1 December 2009
Received in revised form 29 January 2010
Accepted 3 February 2010
Available online 17 February 2010

Keywords:
Expressed protein ligation (EPL)
Cell penetrating peptide
Endotoxin
Intracellular delivery

A B S T R A C T

Expressed protein ligation (EPL) is a useful method for the native chemical ligation of proteins with other proteins or peptides. This study assessed the practicability of EPL in the preparation of fusion proteins of enhanced green fluorescent protein (EGFP) with chemically synthesized cell-penetrating peptides (CPPs) for intracellular delivery. Using intein-mediated purification with an affinity chitin-binding tag (IMPACT) system, the thioester of EGFP (EGFP-SR) was prepared. Optimization of the ligation of EGFP-SR with arginine 12-mer (R12) produced the fusion protein in high yield. The EPL procedure also allows the preparation of EGFP-R12 containing a low level of endotoxin (ET), via the satisfactory ET removal of EGFP-SR prior to ligation with the R12 peptide. Fusion proteins of EGFP with R12 and the α-isomer of R12 prepared by EPL showed similar levels of cellular uptake compared to the fusion protein directly expressed in Escherichia coli.

1. Introduction

Cell penetrating peptides (CPPs) have been employed as vectors for the intracellular delivery of various molecules which have difficulty in entering cells by themselves [1,2]. One of the most desirable applications of CPPs would be the delivery of bioactive proteins having therapeutic prospects [3]. However, contamination of CPP-fusion proteins by the endotoxins (ETs) can be a potential problem when the fusion proteins are expressed in Escherichia coli [4].

ETs (lipopolysaccharides, LPS) are a major component of the outer membrane of Gram-negative bacteria. ETs are known to induce a strong response in the immune system. Disruption of bacterial membranes upon the harvesting of recombinant proteins from E. coli leads to the release of ETs from the membranes and the contamination of ETs in the proteins of interest. Misleading interpretation of the results by the contamination of ETs may be obtained if using immune--associated cells in the assay [5-7]. When ETs enter the blood circulation, they induce various undesired physiological responses, such as the release of proinflammatory materials, fever, coagulopathy, septic shock, and even mortal outcome [8]. Therefore, the preparation of biological products with a sufficiently low ET level is a crucial issue. Since ETs are negatively charged, due to the phosphate groups in lipid A, that is a major component of ETs [9], removal of ET from cationic proteins is not an easy task [10]. Arginine-rich peptides, including oligoarginines and the HIV-1 Tat peptide, comprise a major class of CPPs. These positively charged peptides are employed as CPPs and CPP-fusion proteins are expressed in E. coli, this concern of contamination of the ETs would also be applicable.

Expressed protein ligation (EPL) is a posttranslational splicing of protein segments that accompanies exclusion of an internal segment (Intein) followed by spontaneous attachment of a lateral segment [11]. Intein-mediated EPL has already been employed to form conjugates of proteins or peptides with proteins, peptides, unnatural amino acids, fluorophores and dendrimers [12-16]. This methodology is also promising for the preparation of novel CPP-fusion proteins bearing CPPs with various chemical structures including those composed of non-gene-encoded amino acids (i.e., α-amino acids, etc.). However, only limited information is available on the applicability of this methodology for the preparation of the CPP–protein conjugates [12-16].

In this report, we assessed the practicability of EPL through the preparation of fusion proteins of the enhanced green fluorescent protein (EGFP) with the arginine 12-mer (R12) peptide, a representative oligoarginine CPP [17], as a model. Optimization of the ligation efficiency, procedures to prepare the EGFP–R12 of low ET content, and the internalization efficiency of the obtained EGFP–R12 were studied.
2. Materials and methods

2.1. Reagents

The amino acid derivatives and Rink amide resin were purchased from the Peptide Institute (Osaka, Japan) and Novabiochem (Laufelfingen, Switzerland). Other chemicals unless specifically noted were from Wako Pure Chemicals (Osaka, Japan).

2.2. Synthesis of peptides

CG-R12 [NH₂-Cys-Gly-{Arg}₁₂-amide] and CG-dR12 [NH₂-Cys-Gly-{Arg}₁₂-amide] were synthesized by 9-fluorenymethyloxycarbonyl (Fmoc)-solid-phase peptide synthesis on a Rink amide resin as previously reported [18]. For the preparation of FITC-CG-R12 [FITC-GABA-Cys-Gly-{Arg}₁₂-amide] and FITC-dR12 [FITC-GABA-{Arg}₁₂-amide], a γ-aminobutyryl (GABA) residue was employed as a linker connecting N-terminal FITC moiety to the arginine peptides. Deprotection of the peptides and cleavage from the resin were conducted by the treatment of trifluoroacetic acid/1,2-ethanedithiol (95:5) for 3 h at room temperature. The synthesized peptides were purified by high performance liquid chromatography followed by lyophilization. The fidelity of the synthesized peptides was confirmed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) using a Voyager-DE STR (Applied Biosystems).

2.3. Conjugation of EGFP to synthetic peptide using EPL

2.3.1. Preparation of EGFP-thioester (EGFP-SR)

H6-EGFP, H6-EGFP-R12, and H6-EGFP-CG-R12 were prepared by pET bearing respective H6-EGFP cDNAs. The E. coli BL21 (DE3) transformed with these plasmids was grown in LB medium (800 ml). IPTG (0.1 mM) was employed for the protein overexpression. The H6-EGFP proteins were purified by a Ni-NTA resin column (Qiagen, Hilden, Germany) (1 ml) according to the protocol by the manufacturer. Briefly, E. coli cells were lysed in lysis buffer (50 mM NaH₂PO₄ containing 300 mM NaCl, 10 mM imidazole, and 10 mM 2-mercaptoethanol (pH 8.0)). The lysate was mixed with Ni-NTA resin (1 ml for 800 ml cell culture) at 4 °C for 1 h. The resin slurry was then transferred to a column and washed with washing buffer (50 mM NaH₂PO₄ containing 300 mM NaCl, 20 mM imidazole, and 10 mM 2-mercaptoethanol (pH 8.0)). Finally, bound proteins were eluted from the column with elution buffer (50 mM NaH₂PO₄ containing 300 mM NaCl, 250 mM imidazole, and 10 mM 2-mercaptoethanol (pH 8.0)). The fraction containing the desired protein was repetitively concentrated using a Vivaspin 4 column and diluted with 3× PBS to replace the buffer.

2.5. Endotoxin removal from proteins

2.5.1. Ion exchange columns

Resource S (methyl sulfate) and HiTrap Q HP (quaternary ammonium) pre-packed columns (GE Healthcare, Amersham, UK) (column volume, 1 ml each) operated on the AKTA Explorer system (GE Healthcare) were employed for the removal of ET. H6-EGFP-CG-R12 was diluted in buffer A (20 mM MES containing 100 mM NaCl, pH 6.0) prior to loading onto Resource S. The column was first eluted with buffer A (10 ml) and then with a gradient of 0–100% buffer B (20 mM MES containing 2 M NaCl, pH 6.0) over 20 min (flow rate, 1 ml/min). When HiTrap Q HP was used for the purification of H6-EGFP-CG-R12, the protein was diluted in the buffer (20 mM Tris–HCl containing 50 mM NaCl, pH 8.0) and then loaded onto the column. The column was eluted with the same buffer (flow rate, 0.5 ml/min) and the eluate that contained the desired protein was collected. For the purification of the EGFP-SR, the protein was diluted in buffer A (20 mM Tris–HCl, pH 8.0), loaded on a column of HiTrap Q HP, and eluted with a gradient of buffer B (20 mM Tris–HCl containing 1 M NaCl, pH 8.0) over 20 min at the flow rate of 0.5 ml/min.

2.5.2. Polymyxin B (PMX) column

Removal of the ET was conducted using a Detoxi-Gel Endotoxin removal column (1 ml) (Pierce, Rockford, IL) following the manufacturer’s protocol. The solution of the proteins (H6-EGFP and H6-EGFP-CG-R12 in 50 mM Tris–HCl containing 500 mM NaCl, pH 8.5) was applied to the column equilibrated with the same buffer and left for 1 h at 4 °C. The protein was then eluted from the column by the same buffer. Fractions containing the desired proteins were concentrated using a Vivaspin 4 column prior to the replacement of the
buffer by 3× PBS (for H6-EGFP) and 0.1 M Tris–HCl containing 500 mM NaCl and 10 mM MESNA, pH 8.5 (for EGFP–SR), as described in Section 2.3, respectively.

2.5.3. On-column washing with Triton X-114

The E. coli lysate containing H6-EGFP-CG-R12 (from 800 ml culture) was lysed in the lysis buffer and applied to the Ni-NTA column (1 ml) similar to that described in Section 2.3. The column was washed with 50-bed volumes of washing buffer containing 0.1% Triton X-114 [20] followed by 20-bed volumes of the same buffer containing no Triton X-114. H6-EGFP-CG-R12 was then eluted with elution buffer and the fraction containing H6-EGFP-CG-R12 was repetitively concentrated using a Vivaspin 4 column and diluted with 3× PBS to replace the buffer to yield 10.6 mg of the protein.

For on-column Triton X-114 washing of EGFP-Intein-CBD on the chitin bead column, the lysate containing EGFP-Intein-CBD (from 2400 ml culture) was applied to the chitin bead column (24 ml) as described in Section 2.3.1 prior to washing with 50-bed volumes of 20 mM Tris–HCl containing 500 mM NaCl (pH 8.5) and 0.1% Triton X-114. The column was then washed with 20-bed volumes of 20 mM Tris–HCl containing 500 mM NaCl (pH 8.5) and subjected to on-column cleavage of the EGFP-Intein-CBD at 4 °C as described in Section 2.3.1 to generate the EGFP-SR (6.0 mg).

2.6. Endotoxin assay

The Endospecy LAL kit and endotoxin standard (E. coli O133) were both purchased from Seikagaku Biobusiness (Tokyo, Japan), and the ET contents in the proteins were analyzed by a chromogenic limulus amebocyte lysate (LAL) test following the instructions from the manufacturer. The proteins for the assay and ET standard were titrated in various concentrations using ET-free water. Each titration (40 μl) of the proteins was mixed with an equal volume of freshly prepared LAL reagent in a 96-well microplate. After standing for 30 min at 37 °C, solutions of sodium nitrite, sulfamic acid and N-(1-naphthyl)ethylenediamine were added to the solution. Absorbance of the samples at 570 nm was monitored by a Multiskan FC plate reader (Thermo Scientific, San Jose, CA). The ET level of the proteins was calculated using the ET standard curve.

2.7. Cell culture

Human cervical cancer-derived HeLa cells were purchased from the Riken BRC Cell Bank (Ibaraki, Japan), and cultured in α-minimum essential medium containing 10% heat-inactivated bovine serum [α-MEM(+)] (Invitrogen, Eugene, OR). The cells were grown on 100-mm dishes and incubated at 37 °C under 5% CO₂.

2.8. Flow cytometry

HeLa cells (1 × 10⁵ cells/well) were seeded in a 24-well microplate and cultured in α-MEM(+) for 24 h. The cells were treated with each protein (0.5 μM, 200 μl). The cells were washed three times with 100 μl of 0.5 mg/ml heparin in PBS and then treated with the treatment with 0.1% trypsin at 37 °C for 10 min. The collected cells were pelleted by centrifugation at 700×g for 5 min, washed twice with PBS and finally resuspended in 400 μl of PBS. The cell-associated fluorescence was detected by laser excitation at 488 nm and emission filter at 515–545 nm (10,000 events) on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.9. Confocal laser scanning microscopy (CLSM)

HeLa cells (3 × 10⁵ cells/well) were seeded on 35-mm glass-bottomed dishes (Iwaki, Tokyo, Japan) and cultured in α-MEM(+) for 24 h. After complete adhesion, the cells were washed with α-MEM(+) and treated with 0.5 μM protein in α-MEM(−). After incubation for 12 and 48 h, the cellular localization of the proteins was analyzed without washing and after heparin washing using PBS containing 0.5 mg/ml heparin by the FV300 CLSM system (Olympus, Tokyo, Japan) without fixation.

3. Results and discussion

3.1. Preparation of EGFP-CG-R12 using EPL

Intracellular protein delivery using CPPs usually employs conjugation of proteins with CPPs to ensure efficient delivery, and this is accomplished by the preparation of fusion proteins with CPPs using E. coli expression systems [21–23] or chemical conjugation of these molecules [24,25]. Since the control of chemical processes to yield single forms of the conjugates is often difficult, it seems likely that the former approach is more preferably employed. However, expression of the CPP-fusion proteins is sometimes difficult due to the potential toxicity of the CPPs to E. coli [24], and contamination of the ETs with the CPP-fusion proteins is usually inevitable. The enhanced green fluorescent protein (EGFP) has been extensively employed as a model protein to study the efficiency of the CPP-mediated delivery, distribution into cells and the mechanisms of cellular uptake [21–25], and here we also employed EGFP to assess the applicability of EPL to obtain CPP-fusion proteins of low ET contents.

Intracellular protein delivery using CPPs usually employs conjugation of proteins with CPPs to ensure efficient delivery, and this is accomplished by the preparation of fusion proteins with CPPs using E. coli expression systems [21–23] or chemical conjugation of these molecules [24,25]. Since the control of chemical processes to yield single forms of the conjugates is often difficult, it seems likely that the former approach is more preferably employed. However, expression of the CPP-fusion proteins is sometimes difficult due to the potential toxicity of the CPPs to E. coli [24], and contamination of the ETs with the CPP-fusion proteins is usually inevitable. The enhanced green fluorescent protein (EGFP) has been extensively employed as a model protein to study the efficiency of the CPP-mediated delivery, distribution into cells and the mechanisms of cellular uptake [21–25], and here we also employed EGFP to assess the applicability of EPL to obtain CPP-fusion proteins of low ET contents.

As depicted in Scheme 1A, procedures for the preparation of EGFP-CG-R12 by the IMPACT system, in which the dodecaarginine amide bearing a Cys–Gly segment at the N-terminus was employed as a CPP to ligate the thioester of EGFP, are divided into four steps: (i) expression of EGFP-Intein-CBD by E. coli, (ii) binding of EGFP-Intein-CBD to the chitin column, (iii) induction of the on-bead cleavage between EGFP and Intein-CBD by sodium 2-sulfanylethanesulfonate (MESNA), and (iv) final ligation of R12 to EGFP. EGFP has a lysine at the C-terminus and it was reported that the presence of Lys at this position may accelerate the liberation of EGFP from the EGFP-Intein-CBD by the hydrolisis during the expression in E. coli when cultured at 30 or 37 °C [16]. Therefore, the IPTG induced expression in FINAL ligation of R12 to EGFP. EGFP has a lysine at the C-terminus and it was reported that the presence of Lys at this position may accelerate the liberation of EGFP from the EGFP-Intein-CBD by the hydrolisis during the expression in E. coli when cultured at 30 or 37 °C [16]. Therefore, the IPTG induced expression in our experiment was performed at the lower temperature of 20 °C for 16 h to avoid the possible hydrolisis of the EGFP-Intein-CBD.

The efficiency in the on-column cleavage to generate EGFP-SR was assessed using MESNA. Considering that a higher concentration of MESNA should increase the cleavage efficiency [12], incubation with 100 mM MESNA instead of that listed in the manufacturer’s specification (50 μM) was employed. Incubation was conducted for 24 h by referring to previous reports [12,16]. When the efficiency of the thiolysis of EGFP-Intein-CBD to yield EGFP-SR was analyzed by SDS-PAGE, a considerable amount of the EGFP-Intein-CBD remained uncleaved on the chitin column by the MESNA treatment at 4 °C (Fig. 1, lane 1). On the other hand, almost all of the EGFP-Intein-CBD was cleaved to yield EGFP-SR when treated at 20 °C (Fig. 1, lane 2). The fractions of EGFP-SR obtained after treatment with MESNA at 4 °C and 20 °C showed a single band on SDS-PAGE (Fig. 1, lane 4). Although...
these fractions might have contained a small amount of EGFP where the thiol moiety was hydrolyzed, the fractions of EGFP-SR were subjected to ligation with CG-R12 without further purification at this stage, and the conditions to yield a higher amount of the final compound were explored.

A time-course study of ligation using 50 µM EGFP-SR and 500 µM CG-R12 in 0.1 M Tris–HCl containing 500 mM NaCl and 10 mM MESNA (pH 8.5) suggested that the ligation reached almost completion after 24 h even when incubated at 4 °C (data not shown). Therefore, the incubation time of 36 h was employed in the following experiments to ensure completion of the ligation. To assess the effect of temperature on the ligation, the ligation was conducted at 4, 20 and 37 °C using 50 µM EGFP-SR and 500 µM CG-R12. The ligation yields at the respective temperatures were almost comparable (87–92%) when analyzed by SDS-PAGE, and 20 °C, which yielded the highest amount of product, was thus employed for incubation in the later study. On the other hand, a significant dependence of the ligation efficiency on the EGFP-SR concentration was observed. The ligation yield of EGFP-SR with a 10 molar equivalent of CG-R12, analyzed by SDS-PAGE, was elevated from 77% to 92% with the increase in the EGFP-SR concentration from 10 to 50 µM. No significant aggregation of the proteins was observed even when 50 µM EGFP-SR and 500 µM CG-R12 were employed. The SDS-PAGE after the ligation of 50 µM EGFP-SR with 500 µM CG-R12 at 20 °C is shown in lane 5 of Fig. 1. An intense band corresponding to the desired product EGFP-CG-R12 (upper band) as well as the trace amount of a band that may correspond to EGFP (lower band) formed by the hydrolysis of EGFP-SR during the MESNA treatment of the EGFP-Intein-CBD on the chitin column and during the ligation of EGFP-SR and CG-R12 were observed. The mixture was then subjected to DEAE-Sepharose purification to remove the EGFP and yield EGFP-CG-R12 giving a single band on SDS-PAGE (Fig. 1, lane 6). The mass of EGFP-CG-R12 was further confirmed by MALDI-TOF MS [(M + H)+ 28965.4 (theoretical 28974.9)]. As for the ligation of EGFP-SR with CG-R12, EGFP-SR obtained by the treatment of EGFP-Intein-CBD with MESNA at 20 °C was analyzed from the band densities of the SDS-PAGE (Fig. 1, lane 5) whereas it was 85% for the EGFP-SR obtained from the treatment of the EGFP-Intein-CBD with MESNA at 20 °C. However, considering that a significant amount of the EGFP-Intein-CBD remained uncleaved on the chitin column in the

Scheme 1. Preparation of fusion proteins of EGFP and R12 via EPL and E. coli expression system. (A) Intein-mediated purification with a chitin-binding tag (IMPACT) system used for the preparation of the EGFP-thioester (EGFP-SR). The fusion protein of EGFP with the Intein-chitin binding domain (CBD) segment was expressed in E. coli (EGFP-Intein-CBD). The E. coli cell lysate was loaded onto the chitin column to allow binding of CBD to the chitin bead. On-column treatment of the EGFP-Intein-CBD with MESNA yielded EGFP-SR. Effective removal of ET from EGFP-SR prior to the chemical ligation with CG-R12 yielded EGFP-CG-R12 of a low ET content. (B) The fusion protein bearing the corresponding sequence of EGFP-CG-R12 in (A) but having the 6-His tag on its N-terminus (H6-EGFP-CG-R12) was expressed in the E. coli. The ETs were removed from the H6-EGFP-CG-R12 after purification of the protein on the Ni-NTA column.
former case whereas complete cleavage was attained in the latter, the treatment of EGFP-Intein-CBD with MESNA at 20 °C may attain a higher overall yield of the EGFP-CG-R12.

3.2. Endotoxin removal from the EGFP-R12 fusion proteins

ETs are stable at various pHs and temperatures used for the protein expression from E. coli [8]. Many chromatographic techniques, including ion exchange chromatography and affinity chromatography, such as immobilized histidine, poly-arginine, poly(ethyleneimine) and polymyxin B (PMX), have been employed for the removal of ET [26–28]. However, even after purification through these chromatographies, the complete removal of the ETs is sometimes difficult and the non-specific adsorption of the proteins on the separation columns may accompany a considerable loss of the products [9].

Prior to analyzing the ET contents in the CPP-fusion proteins obtained by EPL, we assessed those in the proteins directly expressed in E. coli. EGFP was also employed as a model of the cargo protein and H6-EGFP (Scheme 1A). Considering that a Cys–Gly sequence is inserted between the EGFP and R12 segments in the fusion protein obtained from the EPL (EGFP-CG-R12 as described above), and that insertion of the 6-His-tag would be beneficial for the purification of the expressed proteins, a protein having H6-EGFP-CG-R12 was designed and expressed in E. coli using the plasmid of the corresponding gene. The H6-EGFP that lacks the CG-R12 segment was also prepared (Scheme 1B).

The H6-EGFP-CG-R12 and H6-EGFP proteins conventionally expressed from the E. coli were purified by a Ni-NTA column and then subjected to the PMX column (Scheme 2A and B). In the case of H6-EGFP, the PMX column was highly effective for the ET removal (Scheme 2A). A single passage of the protein through the column yielded a product of a highly reduced ET level (0.06 EU/µg protein), indicating that the His-tag attached with EGFP was not an obstacle for the ET removal (Table 1a). However, the attachment of the R12 segment with the protein significantly reduced the recovery from the PMX column (Scheme 2B). When H6-EGFP-CG-R12 was applied to the PMX column, there was no substantial recovery of the protein even by the high salt elution using 50 mM Tris–HCl containing 2 M NaCl (pH 8.5) (data not shown). Alternatively, almost 99% of the contaminating ET in H6-EGFP-CG-R12 was removed by the purification on the anionic exchange column (HiTrap Q HP) (Scheme 2b). However, the level of the remaining ET was still relatively high (14 EU/µg) and protein recovery was low (23%) (Table 1b). Even when the protein was repurified again on the same column, there was only a very small difference in the level of the contaminating ET (14 versus 10 EU/µg) together with a significant loss in the proteins (recovery of protein, 35%) (Table 1b′). The purification by the cationic exchanger Resource S (Scheme 2c) yielded similar results; 99% of the ETs was removed by this column to yield the H6-EGFP-CG-R12 fraction containing 19 EU/µg ETs (recovery of protein, 31%) (Table 1c). Repetitive purification using the same column slightly reduced the amount of the contaminating ETs (13 EU/µg) together with a 42% loss of the protein (Table 1c′). Non-ionic detergents including Triton X-114 have been employed for the ET removal of proteins [29], and successful elimination of the ETs from His-tagged proteins by Triton X-114 washing on the Ni-NTA column was reported [20]. However, an unsatisfactory result was obtained by the same protocol to yield H6-EGFP-CG-R12 containing 28 EU/µg ET (Table 1d). The above results suggested the difficulty in removal of the ETs from proteins bearing arginine-rich CPP sequences when the CPP fusion proteins are expressed in E. coli.

On the other hand, when EPL is employed to obtain fusion proteins with the CPPs, and if the ETs are effectively removed prior to the ligation with the CPP segments, this unfavorable contact of the CPP segments with the ETs can be avoided (Scheme 1A). This was confirmed by the following experiments as outlined in Scheme 2C. EGFP-SR, obtained by the treatment of EGFP-Intein-CBD on a chitin column with MESNA (Scheme 1A), was directly applied to the PMX

Scheme 2. Trials for ET-removal from H6-EGFP (A), H6-EGFP-R12 (B), and EGFP-SR (C). The results from the trials shown by the letters in parentheses are listed in Tables 1 and 2.
As expected, highly efficient removal of ETs was achieved by this single purification step to obtain EGFP-SR containing less than 0.1 EU/µg ETs (Table 2f). The protein recovery in this purification step was also more than 80%. In addition, purification using a HiTrap Q column was also effective, but less efficient than the PMX column; EGFP-SR containing 14 EU/µg ETs was obtained with protein recovery of 67% (Table 2f). However, by increasing the amount of proteins charged on the PMX column, the efficiency of the ET removal decreased (Table 2f). In such a case, Triton X-114 washing of the EGFP-Intein-CBD on a chitin column was found to be highly effective. By this washing prior to the MESNA treatment, the ET level in the resulting ET-free CPP-fusion proteins was highly effective, also applicable to the purification of the former sample using the PMX column accomplished the further removal of the ETs to yield EGFP-SR containing 0.08 EU/µg ETs (Table 2). The successive purification of the former sample using the PMX column accomplished the further removal of the ETs to yield EGFP-SR containing 0.08 EU/µg ETs (Table 2). The efficiency of internalization of the prepared CPP-fusion proteins was 100%.

### Table 1

**ET levels of H6-EGFP and H6-EGFP-CG-R12 after ET removal.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Conditions of ET removal</th>
<th>Methods</th>
<th>Loaded protein (mg)</th>
<th>Total ET (EU)</th>
<th>ET (EU/µg) Before</th>
<th>ET (EU/µg) After</th>
<th>Protein recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>H6-EGFP</td>
<td>PMX (a)</td>
<td>1.3</td>
<td>50,000</td>
<td>109</td>
<td>109</td>
<td>0.06</td>
<td>66%</td>
</tr>
<tr>
<td>H6-EGFP-CG-R12</td>
<td>Triton X-114 washing (d)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>19</td>
<td>13</td>
<td>58%</td>
</tr>
</tbody>
</table>

The letters in parentheses correspond to those in Scheme 2. The ET levels of H6-EGFP and H6-EGFP-CG-R12 after ET removal are shown in Table 1.

### Table 2

**ET levels of EGFP-SR after ET removal.**

<table>
<thead>
<tr>
<th>Conditions of ET removal</th>
<th>Methods</th>
<th>Loaded protein (mg)</th>
<th>Total ET (EU)</th>
<th>ET (EU/µg) Before</th>
<th>ET (EU/µg) After</th>
<th>Protein recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-SR</td>
<td>HiTrap Q HP (e)</td>
<td>0.36</td>
<td>2,160,000</td>
<td>14</td>
<td>14</td>
<td>67%</td>
</tr>
<tr>
<td>PMX (f)</td>
<td>0.36</td>
<td>2,160,000</td>
<td>14</td>
<td>0.01</td>
<td>80%</td>
<td></td>
</tr>
<tr>
<td>Combination of Triton X-114 washing and PMX</td>
<td>ND</td>
<td>ND</td>
<td>10,360,000</td>
<td>34</td>
<td>86%</td>
<td></td>
</tr>
</tbody>
</table>

The letters in parentheses correspond to those in Scheme 2. The amount of protein loaded on the PMX column in f was about three times as much as that in e. The ET content in the protein obtained after buffer washing instead of Triton X-114 on the Ni-NTA column was 1500 EU/µg. Yield of the protein obtained after the Triton X-114 wash was comparable to that obtained after the buffer washing; 10.6 mg of H6-EGFP-CG-R12 was obtained from 800 ml of culture after the Triton X-114 washing. The protein recovery (yield in purification) was calculated on the basis of the protein amounts before and after the purifications using the molar extinction coefficient at 488 nm (55,000 M⁻¹ cm⁻¹).

### 3.3. Cellular uptake of the R12- or dR12-fused EGFP

We next examined the efficacy of internalization of the prepared proteins. EGFP fused with the d-isomers of R12 (EGFP-CG-dR12) was on the column strongly interacted with H6-EGFP-CG-R12 to retain it on the column. Therefore, even when an unused PMX column is employed for the R12-bearing proteins partially purified with, for example, ion-exchange chromatography, increasing amount of the loaded samples would easily saturate the PMX moiety on the column with contaminating ETs to hamper the elution of the R12-bearing proteins. We thus conclude that the approach using EPL should be more reliable for the preparation of ET-free CPP-fusion proteins.

**Fig. 2.** Cellular uptake of the fusion proteins of EGFP with the R12 peptides. The HeLa cells were treated with 0.5 µM proteins in α-MEM containing 10% bovine serum. The cells treated with the medium were used as the control. After incubation for 6, 12, 24 and 48 h, the fluorescence derived from EGFP in the cells was analyzed by FACS. The error bars represent the standard deviation (n = 3).
similarly prepared using EPL. The feasibility of ligation with ε-amino acids or non-gene-encoded amino acids is also an advantage of this EPL-employing system.

HeLa cells were treated with EGFP-CG-R12 or EGFP-CG-D-R12 (0.5 µM each) for 6, 12, 24 and 48 h, and the amount of protein taken up by the cells was analyzed by flow cytometry (Fig. 2). H6-EGFP-CG-R12 and H6-EGFP conventionally expressed in *E. coli* were employed as references. In this experiment, proteins of low ET contents as described in Section 3.2 were employed. The efficiency of cellular uptake of EGFP-CG-R12 and EGFP-CG-D-R12 was almost comparable to or slightly higher than that of H6-EGFP-CG-R12. This ensured the ability of the CPP-fusion proteins obtained by the EPL method to internalize into the cells. The amounts of EGFP-CG-R12 and EGFP-CG-D-R12 taken up by the cells were about 7–8 and 5 times higher than those of H6-EGFP after 12 and 48 h, respectively. Employment of the ε-form of R12 (dR12) as CPP of EGFP did not yield a marked difference on the efficiency of cellular uptake compared with its l-isomer (R12). Further assessment using different cargos should be necessary to establish the efficiency of these CPPs. Considering that the chemical cross-link has mainly been employed for the assessment of the internalization efficiency of the CPP-protein conjugates, especially when the CPPs contain non-gene-encoded amino acids, and that

---

**Fig. 3.** Confocal microscopic observation of the HeLa cells treated with fusion proteins of EGFP with R12. The cells were incubated with each protein (0.5 µM) in α-MEM containing 10% bovine serum for 12 and 48 h. The cells were then analyzed using CLSM without washing (left panels) or after heparin washing (right panels). For the heparin washing, the cells were treated with PBS containing 0.5 mg/ml heparin. Scale bar = 20 µm. Red arrows indicate the EGFP-labeled vesicles and the green arrows show the EGFP proteins bound on the plasma membranes.
difficulties are often accompanied in quantitative and site-specific modification of the proteins by chemical ligation, employment of this ligation method using EPL may open a way for the rational and precise evaluation of the abilities of these CPPs as vectors for intracellular protein delivery using the CPP-fusion proteins of unequivocal structures.

During the course of this study, we encountered unexpected results on the cellular uptake of H6-EGFP-R12—this protein was also prepared by E. coli expression, but lacks the CG linker sequence to connect EGFP and the R12 sequences. Cellular uptake of the H6-EGFP-R12 protein was significantly lower than that of H6-EGFP-CG-R12. Therefore, the lack of two amino acids yielded a significant difference in cellular uptake of the proteins. Although further studies are needed to clarify this observation, one possible reason may be the effect of cysteine on the CPP-mediated cellular uptake. It was recently reported by Aubry et al. that CPPs containing free cysteines were more efficiently internalized than the CPPs lacking cysteines via formation of disulfide bridges between the CPPs and cell surface proteins [30]. To examine whether the cellular uptake of the CG-R12 peptide is also more efficient than the R12 peptide, cells were treated with FITC-labeled CG-R12 or R12 peptides (FITC-CG-R12 and FITC-R12) (1 µM each) for 1 and 3 h, respectively. However, no significant difference was observed for their cellular uptake (data not shown). Without bearing the cargo proteins, these peptides may efficiently internalize into cells and the reported accelerating effect of cysteine for cellular uptake was not observed. Without bearing cargo proteins, these peptides may internalize into cells so efficiently that further effects of cysteine on cellular uptake are not observed.

Live cell observation of HeLa cells treated with the above fusion proteins of EGFP and R12 for 3 h using CLSM produced punctate signals (Fig. 3, red arrows). Involvement of endocytosis including macropinocytosis has been suggested [31,32] and these signals should represent the endocytosed proteins. In addition, strong signals of these proteins were observed at the peripheral of the cells (Fig. 3, left, green arrows). Importance of the accumulation of the arginine-rich CPPs to membrane-associated proteoglycans on their cellular uptake has been pointed out, as well as the induction of macropinocytosis [33]. These signals on the peripheral of the cells should reflect the cell surface accumulation of these proteins and these were effectively removed by heparin washing to facilitate the observation of the signals from the proteins taken up by the cells (Fig. 3, right, red arrows). In correlation with the results of the FACS analysis (Fig. 2), protein signals in the cells and on cell surface were much weaker for H6-EGFP-R12 lacking the CG segment than those having the CG segment. Only very low signals were obtained in cells incubated with H6-EGFP lacking the R12 segment.

4. Conclusion

In this study, we illustrated the usefulness of EPL for the preparation of fusion proteins with arginine-rich CPPs of significantly low ET content by avoiding the unfavorable contact of the CPPs with the ETS; our efforts failed to detach the CPPs from the ETS by ion-exchange or PMX columns once the arginine-rich CPPs formed a complex with the ETS. Considering the detrimental effects of contaminating ETs, for example, to induce inflammatory responses in clinical situations employing CPPs fused to therapeutic proteins, this approach may provide a practical way to eliminate the possibility. This approach is also applicable to prepare fusion proteins with CPPs composed of various amino acids including D-amino acids and peptide mimetics. This feature should be beneficial to design protein delivery systems in vivo, using CPPs having targeting ability to, for example, specific organs and tumors. We hope that this report could provide useful information for future design of CPP fusion proteins with more sophisticated properties.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. S.P. is grateful for the JSPS Postdoctoral Fellowship for Foreign Researchers. H.H. is grateful for the JSPS Research Fellowship for Young Scientists.

References


