A Novel Cell-Penetrating Peptide Derived from Human Eosinophil Cationic Protein

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Abstract

Cell-penetrating peptides (CPPs) are short peptides which can carry various types of molecules into cells; however, although most CPPs rapidly penetrate cells in vitro, their in vivo tissue-targeting specificities are low. Herein, we describe cell-binding, internalization, and targeting characteristics of a newly identified 10-residue CPP, denoted ECP32-41, derived from the core heparin-binding motif of human eosinophil cationic protein (ECP). Besides traditional emphasis on positively charged residues, the presence of cysteine and tryptophan residues was demonstrated to be essential for internalization. ECP32-41 entered Beas-2B and wild-type CHO-K1 cells, but not CHO cells lacking of cell-surface glycosaminoglycans (GAGs), indicating that binding of ECP32-41 to cell-surface GAGs was required for internalization. When cells were cultured with GAGs or pre-treated with GAG-digesting enzymes, significant decreases in ECP32-41 internalization were observed, suggesting that cell-surface GAGs, especially heparan sulfate proteoglycans were necessary for ECP32-41 attachment and penetration. Furthermore, treatment with pharmacological agents identified two forms of energy-dependent endocytosis, lipid-raft endocytosis and macropinocytosis, as the major ECP32-41 internalization routes. ECP32-41 was demonstrated to transport various cargos including fluorescent chemical, fluorescent protein, and peptidomimetic drug into cultured Beas-2B cells in vitro, and targeted broncho-epithelial and intestinal villi tissues in vivo. Hence this CPP has the potential to serve as a novel vehicle for intracellular delivery of biomolecules or medicines, especially for the treatment of pulmonary or gastrointestinal diseases.

Introduction

Cell-penetrating peptides (CPPs) are peptides derived from proteins that can transport cargo such as nanoparticles, low molecular weight compounds, other peptides, proteins, and nucleic acids into cells [1]. CPPs may potentially be used during clinical procedures such as gene therapy and cancer treatment, and thus substantial efforts have been made to discover CPPs with suitable carrier properties [1,2].

Most CPPs are rich in positively charged Arg and/or Lys residues, and are internalized after initially interacting with negatively charged cell surface glycosaminoglycans (GAGs), which cluster CPPs on outer membrane surfaces [3,4]. Cell-surface GAGs are complex polysaccharides that participate in cell growth, differentiation, morphogenesis, migration, and bacterial/viral infections. Major vertebrate GAGs include heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS), and hyaluronic acid (HA) [5,6]. It has been shown that syndecan-1, a heparan sulfate proteoglycan (HSPG), accelerates the uptake of cationic CPPs penetratin and octa-arginine into K562 cells [7].

CPPs are usually divided into two groups [1], synthetic peptides such as oligoarginines which penetrate 293T cells [8,9], and peptides derived from natural proteins such as TAT47-57 (GRKKRRQRRRP) from nuclear transcription activator Tat protein (TAT) of human immunodeficiency virus-1, which penetrates various cell types [10]. In the past two decades, 52 CPPs derived from natural proteins that can translocate across cell membranes have been reported [1,11]. Twenty-eight of these CPPs including 15 viral protein-derived peptides, 7 animal modulator-derived peptides, 3 antimicrobial peptides, and 3 toxin-derived peptides have been demonstrated or predicted to interact with cell-surface HS before penetrating plasma membranes [1,11,12]. Most of these heparin-binding CPPs possess consensus heparin-binding motifs XBBXB or XBBBXXB, where B is a basic amino acid and X is any amino acid. These peptides are further classified as cationic or amphipathic peptides.
Heparin-binding CPPs not only require electrostatic interactions, but also use aromatic residues for hydrophobic interactions with target cells [14]. However, little is known about how sequential aromatic and cationic residues affect the interactions of CPPs with cell-surface molecules.

Human eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) are secretory ribonucleases (RNases) released by activated eosinophils [15]. Both ECP (RNase 3) and EDN (RNase 2) possess antiviral and antiparasitic activities [15]. Interestingly, the RNase activity of ECP is much lower than that of EDN [16], although ECP has stronger antibacterial [15,17] and cytotoxic activities [18]. In addition, ECP binds lipopolysaccharides and peptidoglycans tightly [19]. The N-terminal domain of ECP (residues 1–45) retains most of the antimicrobial properties [20]. Boix and colleagues identified residues 1–38 as responsible for the bactericidal activity [21] and found that a cavity created by residues A8–Q14, Y33–R36, Q40–L44, and H128–D130 could bind a HS disaccharide [22]. We have previously reported that cell-surface GAGs, especially HSPGs, act as receptors to promote ECP internalization via the macropinocytic pathway [23], resulting in apoptosis in Beas-2B cells [24]. The cytotoxicity of ECP was significantly reduced in mutant cell lines that lacked cell-surface HS or GAGs [23]. A sequential segment of ECP, RYRWRCK [25], was subsequently identified as a core heparin-binding motif [25].

Very few CPPs derived from heparin-binding regions in proteins have been reported. Here two 10-residue peptides, ECP<sub>32–41</sub> (RYRWRCKNQN) containing a novel heparin-binding motif of ECP, and EDN<sub>32–41</sub> (NYQRRCKNQN) possessing a consensus heparin-binding motif in EDN [25], were synthesized and their cell-binding, GAG-binding, cell-penetrating, and cargo-transport activities were analysed. Interestingly, only ECP<sub>32–41</sub> displayed CPP-like properties. The main endocytic routes for ECP<sub>32–41</sub> internalization were found to be temperature-sensitive and energy-dependent. ECP<sub>32–41</sub> was able to deliver a small fluorescent molecule, a recombinant protein, and a peptidomimetic drug into cells. Moreover, an ECP<sub>32–41</sub>-tagged protein was preferentially routed to broncho-epithelial and intestinal villi tissues in rat. Here we demonstrate that ECP<sub>32–41</sub> is the first heparin-binding CPP derived from a secretory human RNase, and we propose that it may serve as a new vehicle for intracellular cargo delivery and tissue targeting. It is a promising candidate for further molecular and cellular engineering investigations.

**Results**

**ECP<sub>32–41</sub> Internalization**

Internalization of FITC-ECP<sub>32–41</sub> and FITC-EDN<sub>32–41</sub> was measured as the median fluorescence intensity (MFI) of 6.0 × 10<sup>5</sup> Beas-2B cells that had been treated with one of the FITC-labelled peptides (1 to 20 μM) at 37°C for 1 h, and then treated with trypsin to remove surface-bound peptides. FITC-ECP<sub>32–41</sub> internalization was concentration dependent (Figure 1A), and at each concentration tested, the signal arising from FITC-EDN<sub>32–41</sub> fluorescence was similar to that of the corresponding FITC control (Figure 1A). When Beas-2B cells were treated with 5 μM of a FITC-peptide at 37°C, the fluorescent signal for FITC-ECP<sub>32–41</sub> increased within 5 min, and reached plateau at 30 min (Figure 1B). FITC-EDN<sub>32–41</sub> penetrated the cells to a lesser extent during the 60 min incubation (Figure 1B). After addition of 5 μM ECP<sub>32–41</sub>, intercellular fluorescence was clearly detected 5 and 60 min later by CLSM, whereas a signal for intracellular EDN<sub>32–41</sub> was not detected even after 1 h (Figure 1C). ECP<sub>32–41</sub> therefore penetrated Beas-2B cells in a time- and concentration-dependent manner.

**Figure 1. Internalization of ECP<sub>32–41</sub> and EDN<sub>32–41</sub>.** (A) Beas-2B cells were incubated with 1, 5, 10, or 20 μM FITC-ECP<sub>32–41</sub>, FITC-EDN<sub>32–41</sub>, or FITC at 37°C for 1 h. The cells were washed twice with 500 μl PBS, trypsinized at 37°C for 15 min, suspended in 500 μl PBS, and then subjected to flow cytometry. (B) Beas-2B cells were incubated with 5 μM FITC-ECP<sub>32–41</sub>, or FITC at 37°C for 5, 10, 30, or 60 min. The cells were then treated as described in (A) and subjected to flow cytometry. The results in (A) and (B) are expressed as the mean ± standard deviation (S.D.), n = 3. (C) Beas-2B cells were incubated with 5 μM FITC-ECP<sub>32–41</sub>, FITC-EDN<sub>32–41</sub>, or FITC at 37°C for 5 or 60 min.
removal of amino acids from the expected, differences in the internalization were observed with the sequence (Table 2). Individually carried out done from PLOS ONE | www.plosone.org 3 March 2013 | Volume 8 | Issue 3 | e57318 therefor be ascribed to both the W3R4 dipeptide sequence, and to Beas-2B cells than did ECP32–41 and ECP32–41R3Q after 1 h internalization. Surprisingly, twice as much ECP32–41W4R bound to Beas-2B cells as ECP33–41 and ECP34–40 showed significantly 44%, 28% and 61% internalization. The functionality of ECP 32–41, as a CPP, could not be ascribed to both the W3R4 dipeptide sequence, and to Beas-2B cells than did ECP32–41 and ECP32–41R3Q after 1 h internalization. Surprisingly, twice as much ECP32–41W4R bound to Beas-2B cells as ECP33–41 and ECP34–40 showed significantly 44%, 28% and 61% internalization. The functionality of ECP 32–41, as a CPP, could therefore be ascribed to both the W3R4 dipeptide sequence, and a nearly complete heparin-binding motif.

Table 1. Sequences and molecular weights of peptides.

| Peptide | Sequence | Molecular weight (Da) |
|---------|----------|----------------------|
| ECP32–41 | NYRWRCKNQN | 1381 |
| EDN32–41 | NYQRRCKNQN | 1323 |
| ECP32–41R3Q | NYQWRCKNQN | 1353 |
| ECP32–41W4R | NYRWRCKNQN | 1351 |
| ECP33–41 | YRWRCKNQN | 1268 |
| ECP32–40 | YRWRCKNQN | 1267 |
| ECP32–39 | YRWRCKNQ | 1153 |
| ECP32–38 | RWCKNQN | 1104 |
| ECP32–38 | NWRCK | 1025 |
| TAT37–57 | GIKRRQRRRP | 1493 |
| KLA | KLALALKLALALKL | 1524 |
| KLA-TAT37–57 | KLALALKLALALKLGRKRRQRRRP | 2999 |
| KLA-ECP32–41 | KLALALKLALALKNYRWRCKNQN | 2887 |

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Table 2. Binding and penetrating activities of synthetic peptides in Beas-2B cells.

| Peptide | Binding (%)a | Penetrating (%)b |
|---------|--------------|------------------|
| ECP32–41 | 100 | 100 |
| EDN32–41 | 19.4 ± 0.05*** | 71.0 ± 4.24*** |
| ECP32–41R3Q | 87.2 ± 0.40 | 70.6 ± 3.30* |
| ECP32–41W4R | 549.9 ± 1.00*** | 32.3 ± 6.55** |
| ECP33–41 | 54.7 ± 3.37* | 48.2 ± 2.48* |
| ECP32–40 | 72.4 ± 4.68* | 70.8 ± 4.97* |
| ECP34–41 | 39.3 ± 2.76** | 34.3 ± 2.41** |
| ECP32–39 | 82.6 ± 3.20 | 35.0 ± 6.98** |
| ECP32–38 | 86.6 ± 3.75 | 28.7 ± 6.24** |

XX amino-n-butylacat. ND: not determined. The result is expressed as the mean ± S.D., n=4.

Effects of GAG on ECP32–41 Binding

Cell-membrane GAGs including HS, CS/DS, and HA are necessary for CPP internalization [5,6]. Effects of GAGs on ECP32–41 cellular binding ability, soluble GAGs including LMWH, CSC, and HA were used as competitors to inhibit the attachment of ECP32–41 to Beas-2B cells. At concentrations between 0.01 and 1 μg/ml, LMWH and CSC prevented approximately 70% of the normal ECP32–41 binding, whereas 53% inhibition was observed for CHO-pgsD677 cells normalized to 100%. Beas-2B cells were incubated with 5 μM FITC-peptide at 4°C for 1 h, washed twice with PBS, and subjected to ELISA. The amount of FITC-ECP32–41 bound to Beas-2B cells was normalized to 100%. Beas-2B cells were incubated with 5 μM FITC-peptide at 37°C for 1 h. The cells were washed twice with 500 μl PBS, trypsinized at 37°C for 15 min, suspended in 500 μl PBS, and then subjected to flow cytometry. The fluorescence of cells treated with ECP32–41 was set as 100%. Uptake, strongly suggesting that the length of our ECP-derived CPP was critical for internalization and residues from 32 to 41 were required.

Effects of GAGs on ECP32–41 Binding

Cell-membrane GAGs including HS, CS/DS, and HA are necessary for CPP internalization [5,6]. To assess the effect of GAGs on ECP32–41 cellular binding ability, soluble GAGs including LMWH, CSC, and HA were used as competitors to inhibit the attachment of ECP32–41 to Beas-2B cells. At concentrations between 0.01 and 1 μg/ml, LMWH, CSC, and HA inhibited ECP32–41 binding, with LMWH being the most effective and HA being the least. At concentrations exceeding 50 μg/ml, LMWH and CSC prevented approximately 70% of the normal ECP32–41 binding, whereas 53% inhibition was observed for 100 μg/ml HA (Figure 2A). Hence HS and CS might be involved in ECP32–41 binding to Beas-2B cells. To clarify the roles of cell-surface HS and CS, the binding of ECP32–41 to wild-type and two mutant strains of Chinese hamster ovary (CHO) cells was assessed by ELISA. CHO-pgsD677 cells do not express N-acetylgalcosaminyltransferase and glucurononyltransferase, and therefore lack HS, but produce three times more CS than wild-type CHO-K1 cells [26]. CHO-pgsAV745 cells are deficient in xylosyltransferase so that no GAG was present on the surface [27]. The amount of ECP32–41 bound to CHO-pgsAV745 cells was found to be 52% less than that bound to CHO-K1 cells, suggesting that GAG was required for binding (Figure 2B). Additionally, a 31% reduction in ECP32–41 binding was observed for CHO-pgsD677 cells, even though the cells expressed much more CS than CHO-K1 cells (Figure 2B). GAGs, and especially HSPGs, are therefore crucial for the initial interaction of ECP32–41 with cell surfaces.
ECP32–41 Internalization

Temperature and Energy Dependences of ECP32–41 Internalization

CPPs enter cells by two routes: direct translocation through lipid bilayers or energy-dependent vesicular mechanisms referred to as endocytosis [28]. Direct CPP translocation is usually observed when the CPP concentration is above 10 μM [28]. To characterize the mechanism(s) of ECP32–41 internalization at low concentrations (≤3 μM), we investigated the effect of cellular ATP depletion and low incubation temperature—both of which were expected to inhibit endocytosis. FITC-ECP32–41 internalization was inhibited by 76% at 4°C, compared to 37°C (Figure 4A), when cell samples were first incubated at these temperatures for 30 min prior to addition of 5 μM FITC-ECP32–41. Pre-incubation with sodium azide and deoxyglucose, which depleted the cellular ATP pool, inhibited FITC-ECP32–41 internalization by 57%. ECP32–41 internalization is therefore, temperature- and energy-dependent, indicating that, at low concentrations of ECP32–41, the main internalization route is endocytic in nature.

ECP32–41 Internalization via Lipid-raft Dependent Macropinocytosis

Endocytic pathways are generally grouped into four categories: clathrin- and caveolin-mediated pathways, macropinocytosis, and other less-well characterized clathrin- and caveolin-independent mechanisms [29]. Some of these pathways are also lipid-raft dependent [29]. We pretreated Beas-2B cells with endocytic inhibitors to identify the pathways involved in ECP32–41 internalization. Chlorpromazine, an inhibitor of clathrin-mediated endocytosis, did not affect FITC-ECP32–41 internalization (Figure 4B), suggesting that clathrin-mediated endocytosis was not involved. The lipid-raft pathway inhibitors methyl-β-cyclo-dextrin and genistein inhibited FITC-ECP32–41 internalization by 48% and 40%, respectively. Cellular uptake of ECP32–41 reduced 50% in the presence of filipin III which depleted lipid raft on cell membrane, also suggesting that lipid raft-dependent endocytosis was involved in ECP32–41 internalization. Nocodazole and cytochalasin D, which blocked cytoskeleton polymerization and consequently phagosome and macropinosome formation, respec-
Figure 3. HS-dependent ECP32–41 internalization. (A) Beas-2B cells were treated with the indicated concentrations of LMWH, CS, or HA for 30 min prior to incubation with 5 μM FITC-ECP32–41 at 37°C for 1 h. The cells were washed twice with 500 μl PBS, trypsinized at 37°C for 15 min, suspended in 500 μl PBS, and subjected to flow cytometry. The result is expressed as the mean ± S.D., n = 3. (B) Samples of wild-type and mutant CHO cells were each incubated with 5 μM FITC-ECP32–41 at 37°C for 1 h and then subjected to flow cytometry. The fluorescence of the untreated cells was set to 100%. The result is expressed as the mean ± S.D., n = 3. (C) Samples of wild-type and mutant CHO cells were incubated with 5 μM FITC-ECP32–41 at 37°C for 1 h, then washed twice with 1 ml PBS, and fixed for CLSM. Nuclei were stained with Hoechst 34850. Scale bar: 20 μm. (D) Beas-2B cells were treated with heparinase I, heparinase III, or chondroitinase ABC for 2 h prior to incubation with 5 μM FITC-ECP32–41 at 37°C for 1 h. The cells were washed twice with 500 μl PBS, trypsinized at 37°C for 15 min, suspended in 500 μl PBS, and subjected to flow cytometry. Untreated cells served as the controls. The fluorescence of cells treated with FITC-ECP32–41 was set to 100%. The result is expressed as the mean ± S.D., n = 3. **, P<0.01 and ***, P<0.001.

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Figure 4. Internalization pathway of ECP32–41. (A) Beas-2B cells were incubated at 37°C, 4°C, or with ATP depletion at 37°C for 30 min and then incubated with 5 μM FITC-ECP32–41 for 1 h. The cells were washed twice with 500 μl PBS, trypsinized at 37°C for 15 min, suspended in 500 μl PBS, and subjected to flow cytometry. The fluorescence of cells treated with ECP32–41 was set to 100%. The result is expressed as the mean ± S.D., n = 3. **, P<0.001; ***, P<0.001. (B) Beas-2B cells were incubated with the indicated endocytic inhibitors at 37°C for 30 min, followed by addition of 5 μM FITC-ECP32–41 at 37°C for 1 h. Cells were then treated as described in (A). The result is expressed as the mean ± S.D., n = 3. *, P<0.05; **, P<0.01. doi:10.1371/journal.pone.0057318.g004

Cytotoxic Effects of ECP32–41

To get a comprehensive analysis of toxic profiles induced by ECP32–41, cytotoxic and membrane disruptive properties of ECP32–41 were analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and lactate dehydrogenase (LDH) leakage assay, respectively. Beas-2B was treated with ECP32–41 up to 100 μM at 37°C for 24 h. No sign of any negative effects in cell viability were observed after treatment with ECP32–41 (Figure 5A) and no significant changes (P>0.05) in LDH levels were found between ECP32–41 treated and untreated cells (Figure 5B). These results demonstrated that treatment of cells with ECP32–41 had no effects on cytotoxicity and membrane disruption.

In vitro Delivery of Proteins and Peptides by ECP32–41 into Cells

The ability to mediate cellular uptake of normally impermeable small molecules, proteins, and peptides is an important functional characteristic of CPPs [28]. To determine what type(s) of cargo ECP32–41 could deliver, first, eGFP (28 kDa) was fused to ECP32–41 so that internalization of ECP32–41 could be monitored by flow cytometry. A fluorescent signal shift was clearly observed after incubating Beas-2B cells with eGFP-ECP32–41, indicating that ECP32–41 successfully delivered eGFP into the cells (Figure 6A).

To determine ECP32–41 penetration into the cell in terms of time and intracellular localization, the cytosolic and endosomal fractions of Beas-2B cells were isolated by subcellular fractionation after treatment with eGFP-ECP32–41. Beas-2B cells were incubated with eGFP or eGFP-ECP32–41 (20 μM) at 4°C for 1 h and then shifted to 37°C for further incubation for 1 h, 2 h, 3 h and 4 h separately. Cells were homogenized and fractionated by floatation in Percoll gradients separating cytoplasmic and endosomal fractions. Neither eGFP-ECP32–41 nor eGFP signal was detected along with Actin in cytoplasm even after 4 h incubation (Figure S1A). In terms of endosomal fraction, eGFP-ECP32–41 signal was detected along with LAMP-1 in endosomal fraction after 1 h incubation, the accumulated amount reached maximum at 2 h and then gradually decreased (Figure S1B). In contrast, eGFP signal was not detected even after 4 h treatment. These results suggested that larger cargo (eGFP-ECP32–41) remained in endocytic vesicles for at least 4 h.

Additionally, reduced FITC-ECP32–41 internalization by 48% and 56%, respectively. Dimethyl amilorides, an inhibitor of the Na+/H+ ion exchange pump resulting in the cessation of macropinocytosis, and wortmannin, an inhibitor of both macropinocytosis and clathrin-mediated endocytosis, inhibited internalization by 50% and 53%, which indicated that macropinocytosis was involved. Lipid rafts are therefore involved in ECP32–41 internalization, and two pathways appear to govern ECP32–41 internalization: actin-dependent endocytosis and lipid-raft macropinocytosis.
In general, CPPs should not be cytotoxic when serving as viable delivery vehicles, the effect of ECP32–41 on cell viability was assayed using MTT assay with a well characterized CPP, TAT47–57, as a control [10]. At 100 μM, neither peptide affected cell viability (Figure 5A, Figure 6B); thus ECP32–41, unlike full-length ECP [23,24], could potentially serve as a delivery vehicle. To assess the ability of ECP32–41 to deliver a small cargo, a peptidomimetic drug, KLA, which contained a proapoptotic domain that induces mitochondrial swelling but did not affect the plasma membrane, was chosen as the cargo [30,31]. The cytotoxicity of KLA-TAT47–57 and KLA-ECP32–41 in Beas-2B, A549, Caco-2 and AGS cells were summarized in Table 3. For the lung cell lines, the EC50 values of KLA-ECP32–41 and KLA-TAT47–57 on Beas-2B and A549 cells were quite similar (5 to 7 μM). However, for the digestive-track cell lines, EC50 values of KLA-ECP32–41 were respectively 1.6- and 2.42-fold higher than those of KLA-TAT47–57 in Caco-2 and AGS cells, presumably due to higher expression of HSPGs on lung cells or higher KLA resistance of gastrointestinal cell lines.

Figure 5. Cytotoxicity and membrane disruption by ECP32–41. Beas-2B cells were grown in serum-free medium in the presence of ECP32–41 at indicated concentrations for 24 h. (A) The cytotoxic effect of ECP32–41 was measured by MTT assay. The cell viability of untreated cells was set to 100%. Cells treated with 0.1% Triton X-100 in medium was defined as 100% leakage and LDH released from untreated cells was set as 0% leakage. The result is expressed as means ± S.D., n = 3, no significance (n.s.).

In vitro analysis, a newly identified CPP corresponding to residues 32–41 of human ECP (ECP32–41) was characterized. ECP32–41 delivered a small, fluorescent compound (Figure 1), a recombinant protein (Figure 6A), and a peptidomimetic drug (Figure 6D) into Beas-2B cells, and targeted specific rat tissues (Figure 6A), which was quite similar to tissue distribution as detected in broncho-epithelial and intestinal villi tissues (Figure 7A, 7C), which was quite similar to tissue distribution as detected in broncho-epithelial and intestinal villi tissues. As is known that mammalian mucosal cells are rich in HSPGs, ECP32–41 may potentially be used for in vivo targeting of broncho-epithelial and intestinal villi tissues.

**Tissue Targeting of ECP32–41 in an Animal Model**

GAG expression is related to cell differentiation and growth [32], and specific HPSGs are differentially expressed in different cell types [33]. To delineate tissue targeting by ECP32–41 in vivo and to develop potential applications, eGFP-ECP32–41 and eGFP were separately injected into the circulatory system of specific-pathogen-free rats through tail veins. The tissues were immuno-histochemically stained with anti-eGFP antibody. Interestingly, 1 h after injection, significant eGFP-ECP32–41 signals were detected in broncho-epithelial and intestinal villi tissues (Figure 7A, 7C), which was quite similar to tissue distribution as ECP [23], eGFP alone was not detected in these tissues (Figure 7B, 7D). As is known that mammalian mucosal cells are rich in HSPGs, ECP32–41 may potentially be used for in vivo targeting of broncho-epithelial and intestinal villi tissues.

**Discussion**

CPPs are a class of peptides differing in sequence, size, and charge that can translocate across plasma membranes. In this study, a newly identified CPP corresponding to residues 32–41 of human ECP (ECP32–41) was characterized. ECP32–41 delivered a small, fluorescent compound (Figure 1), a recombinant protein (Figure 6A), and a peptidomimetic drug (Figure 6D) into Beas-2B cells, and targeted specific rat tissues in vivo (Figure 7), showing that it can act as a delivery vehicle in both types of environments.

ECP is a multifunctional protein with ribonucleolytic, cytotoxic, membrane-disrupting, antibacterial, antiparasitic, antiviral, heparin-binding, and cell-penetrating activities. Boix and colleagues identified the heparin-binding residues in ECP as A8–Q14, Y33–R36, Q40–L44, and H128–D130 [22], and we have previously shown that residues 34–38 comprise a critical heparin-binding sequence, and substitution of residues in the ECP sequence 34RWRCK38 with alanine resulted in decreased cell-penetrating activity [25]. Here ECP32–41 is identified as the first CPP derived from a human RNase sequence.
Previous reports have suggested that the guanidinium group of arginine rather than lysine or histidine side-chains is necessary for CPP activity [34,35]. Addition of tryptophan to heptaarginine peptide increases its uptake efficiency [36]. And further, cellular internalization of tryptophan distributed along (RWRRWRRWRRWR) shows higher efficiency than heptaarginine with tryptophan at the N-terminus [37]. Interestingly, although both ECP32–41 and EDN32–41 possess heparin-binding sequences differing only at two positions, they have very dissimilar cell-binding and internalization activities. ECP32–41R3Q and ECP32–41 bound Beas-2B cells similarly (Table 2), but ECP32–41R3Q did not penetrate cells as ECP32–41 did (Table 2). Additionally, although ECP32–41W4R had the strongest affinity for Beas-2B cell among those peptides tested (Table 2), it did not penetrate the cells (Table 2), possibly due to its tight binding to cell-surface GAGs [38]. Residues R3 and W4 in ECP32–41 thus appeared to be crucial for internalization. The two arginines adjacent to W4 in ECP32–41 possibly interacted with negatively charged cell-surface HSPGs, thereby promoting binding. Taken together, the positively charged arginines and the aromatic tryptophan are necessary for ECP32–41 internalization.

Most viral-derived CPPs are rich in basic amino acids [1]. For example, flock house virus coat peptide (residues 35–49, RRRRRNRTRRNRRRVR) is extensively used as a CPP, it can interact with sulfated proteoglycans and negatively charged cell-membrane phospholipids [39]. Interestingly, internalization of the amphipathic peptide penetratin (RQIKIWFQNRRMKWKK) requires electrostatic interactions between basic residues and HS and the presence of aromatic residues, especially tryptophan, for insertion into a lipid bilayer [40]. Although the length of ECP32–41 is comparable to that of TAT47–57 and the antimicrobial CPP SynB3 (RRRLSYSRRRF), physical characteristics of these three CPPs differ. TAT47–57 and SynB3 both have pi values of 12, owing to the presence of many cationic residues, whereas ECP32–41 has only two arginines and one lysine with a pi value of 10.05.

### Table 3. Half maximal effective concentration of KLA-TAT47–57 and KLA-ECP32–41.

| Cell line | EC50 (μM) |
|-----------|-----------|
|           | KLA-TAT47–57 | KLA-ECP32–41 |
| Beas-2B   | 5.64±0.37   | 6.08±0.26   |
| A549      | 6.84±0.13   | 7.17±0.39   |
| Caco-2    | 21.79±0.63  | 35.07±0.77  |
| AG5       | 24.67±5.54  | 59.75±6.82  |

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Interestingly, the percentage of basic residues (50\%) in CyLoP-1 (CRWRWKCCKK) from crotamine [41], one of the main toxins in rattlesnake venom, is similar to that of ECP32–41 (40\%). A consensus motif RWRXK (where X is any amino acid) is present in both CyLoP-1 [41] and ECP32–41. Likewise, internalization of both CyLoP-1 and ECP32–41 requires positively charged residues and non-polar residues, especially tryptophan [41]. Moreover, tryptophan residues are preferentially oriented parallel to the membrane and required for membrane penetration of CPP [42]. ECP32–41, located in a flexible loop structure in intact ECP [22], showed random coil property as determined by circular dichroism spectroscopy (data not shown). Flexibility of structure has been suggested as a favorable property for direct membrane penetration for CPPs, as it might allow efficient cell entry [34,40]. In summary, the presence of aromatic W4, the binding by cationic R3 and R5 to cell surface GAGs, and flexible backbone structure probably all contribute to ECP32–41 internalization.

Three lines of direct evidence indicated that cell-surface HS was involved in ECP32–41 endocytosis. First, soluble HS significantly decreased FITC-ECP32–41 internalization into Beas-2B cells (Figures 2A, 3A). Second, cell-surface HSPGs facilitated FITC-ECP32–41 binding because wild-type CHO cells uptook more FITC-ECP32–41 than did HS- and GAG-deficient CHO cells (Figures 2B, 3B). Third, removal of cell surface HS reduced FITC-ECP32–41 internalization into Beas-2B cells (Figures 3C, 3D). Therefore, cell surface HSPGs mediate ECP32–41 internalization.

In general, three steps are involved in CPP internalization: CPPs first bind to cell surface GAGs, then they move through the cell membrane, and finally they are released into the cytoplasm [43]. Initially, CPPs were thought to bind and directly cross plasma membranes via a receptor- and energy-independent path [44,45]. However, internalization mechanisms, in addition to direct translocation, e.g., clathrin-mediated, caveolin-mediated, macropinocytotic, and clathrin- and caveolin-independent endocytosis, have been reported [29]. Moreover, most CPPs employ two or more internalization pathways [44]. We have previously demonstrated that ECP internalization into Beas-2B cells occurs via HS-facilitated and lipid raft-dependent macropinocytic routes [23]. We have now found that HSPGs act as receptors or attachment factors for ECP32–41 internalization (Figure 3). Additionally, ECP32–41 internalized at 4°C, albeit with a lower efficiency than at 37°C, and endocytotic inhibitor screening suggested that lipid raft-dependent macropinocytic routes were also involved (Figure 4B). The internalization routes of ECP and
ECP\(^{32-41}\) therefore, appear to be similar [23]. However, nocodazole blocked internalization of only ECP\(^{32-41}\) (Figure 4B) but not that of ECP [23], thus multi-endocytic routes should be involved in ECP\(^{32-41}\) internalization.

Previous studies have emphasized that the use of CPPs should improve drug delivery to cells, although CPPs usually target cells promiscuously [46]. Most CPPs have high internalization rates \textit{in vitro} but low target specificity \textit{in vivo} [47]. Certain peptides, denoted cell-targeting peptides, specifically target a certain type(s) of cell(s) and bind to their target(s) strongly [48]. CPP fusion with cell-targeting peptide might therefore, prove useful as drug delivery systems, although however, TAT linked to antibody did not retain the cell-targeting ability of the antibody [46]. Nevertheless, Kuniyasu and colleagues, using phage display technology, isolated the peptide, CAYHRLRRC that contained a lymph node-homing sequence (CAY) and a cell-penetrating motif (RLRR) [49], which selectively penetrated leukaemia and lymphoma cells \textit{in vivo}. Notably, we found that ECP\(^{32-41}\) could penetrate cells \textit{in vitro} and selectively penetrate broncho-epithelial and intestinal villi tissues \textit{in vivo} (Figure 7). ECP\(^{32-41}\) targets specific cells and tissues effectively and thus may be used in the development of innovative biomaterials for molecular detection and diagnosis purposes.

Most known CPPs are non-human in origin, which means that the adaptive immune response to these molecules will be of particular concern during the development of biomedical applications, especially if the CPP is conjugated to a protein or nanoparticle. The overall sequence identity among 13 primate eosinophil RNases, each containing approximately 130 amino acids, is 67%. The sequence identities for ECP\(^{32-41}\) and the correspondent regions of human RNase 2 and RNase 8 are 80% and 50%, respectively, and those for other human RNases are smaller (Table S1). Interestingly, the corresponding 10-amino acid sequences of eosinophil RNases from higher primates, \textit{Pan troglodytes} and \textit{Gorilla gorilla}, the closest living relatives to humans [50], are identical to that of ECP\(^{32-41}\). In addition, the corresponding sequences in \textit{Macaca fascicularis} and \textit{Macaca nemestrina} RNases are 80% identical to ECP\(^{32-41}\) but are completely identical to that of EDN\(^{32-41}\) (Table S2). Therefore, residues 32–41 in ECP may have evolved from those in EDN. Apparently, residues 32–41 are not conserved in the members of the human RNase A superfamily, but represent a specific motif present in higher primates.

In summary, ECP\(^{32-41}\) is not cytotoxic and can be covalently coupled to many different molecules; it has a substantial cargo delivery potential as an attractive candidate for intracellular delivery of therapeutic molecules. GAG-mediated internalization may be the major pathway for ECP\(^{32-41}\) internalization. Finally, ECP\(^{32-41}\) is a human-derived peptide and specifically targets certain tissues, we expect that, with or without modification, it can be useful as a drug delivery system.

### Methods

#### Peptide Design and Synthesis

Peptides with or without an \(N\)-terminally conjugated fluorescein isothiocyanate (FITC) group (Table 1) were synthesised by Genemed Synthesis Inc. and their purities (>90%) were assessed by analytical high-performance liquid chromatography. FITC was conjugated to \(N\)-terminus of ECP\(^{32-41}\) through a 3-carbon linker, which gave a spacer of approximately 10 angstroms in length. Peptide sequences were confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry in Genemed Synthesis Inc.

#### Cell Cultures

Beas-2B cells were cultured in RPMI 1640 medium, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) Glutamine-Penicillin-Streptomycin (Biosera). Chinese hamster ovary (CHO) and AGS cells were maintained in Ham’s F-12, 10% heat-inactivated FBS, 1% (v/v) Glutamine-Penicillin-Streptomycin. A549 cells were cultured in Dulbecco’s Modified Eagle Medium, 10% heat-inactivated FBS, 1% (v/v) Glutamine-Penicillin-Streptomycin. Caco-2 cells were grown in minimum essential medium, 10% FBS, 1% non-essential amino acids, 1% t-glutamine (Biowhittaker), 1% (v/v) Glutamine-Penicillin-Streptomycin. All cells were grown in a 5% CO\(_2\), humidified atmosphere at 37°C. Cell culture media, non-essential amino acids, and FBS were purchased from Invitrogen. Beas-2B, CHO, AGS, A549 and Caco-2 cells were purchased from ATCC.

#### Cell-based ELISA

Cells (\(2\times10^4/well\)) were seeded into 96-well black plate and incubated under a 5% CO\(_2\) atmosphere at 37°C for 24 h. Each well was then washed with 150 μl ice-cold PBS. To prevent non-specific antibody binding, BSA was used as a blocking agent, and PBS containing 2% (w/v) BSA was added to each well at 4°C and incubated for 1 h. The wells were then washed with 100 μl ice-cold PBS, FITC-conjugated peptides were diluted to 10, 20, or 100 μM in PBS and then, medium (95 μl) and a peptide/PBS solution (5 μl) were gently mixed, added into a well, and the plate was placed on ice for 1 h. Each well was then washed with 100 μl PBS and the FITC fluorescent intensity for each sample was measured using a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and excitation and emission wavelengths of 485 nm and 521 nm respectively.

#### Flow Cytometry

Cells (\(3.0\times10^4/well\)) were added into six-well plates and cultured in the indicated medium. After 24 h, each FITC-peptide, dissolved in medium, was added into a well and the samples incubated for 1 h. Cells were then harvested, washed, and suspended in PBS. The fluorescent intensities of the cell samples were measured using a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and excitation and emission wavelengths of 488 nm and 515–545 nm respectively. The relative internalization of each peptide is reported as the mean fluorescence signal for 10,000 cells.

#### Confocal Laser-scanning Microscopy (CLSM)

Cells were cultured on coverslips (\(1.0\times10^4/coverslip\)) in indicated medium. After 24 h, cell samples were each incubated at 37°C for 1 h with an FITC-peptide. The cells were then washed twice with PBS, fixed with 2% (v/v) paraformaldehyde and incubated, first in PBS for 15 min, then with 50 mM NH\(_4\)Cl in PBS for 10 min, and finally permeabilised with 0.5% (v/v) Triton-X-100 at 25°C for 5 min. Nuclei were stained with Hoechst 33342 Fluorescent Stain, (Sigma-Aldrich) during the final 5 min of incubation. Cells were then washed twice with 0.05% Triton-X-100, once with PBS, and the coverslips were mounted in a Vectashield anti-fade mounting medium (Vector Labs). CLSM was performed using LSM510 META (Carl Zeiss, Göttingen, Germany) to assess the distribution of the FITC-peptides in the cells.

#### GAG Competition Assay

Beas-2B cells (\(2\times10^4/well\)) in RPMI 1640 medium were seeded into 96-well black plate for cell binding test, while cells (\(3.0\times10^4/\))
well) incubated in the wells of six-well plates were used for internalization test. After 24 h, cells were treated for 30 min with 0.01, 0.1, 1, 5, 10, 50, or 100 µg/ml of low molecular weight heparin (LMWH; average Mr ~3,000), chondroitin sulfate C (CSC; average Mr ~50,000–58,000), or HA (Mr ~3,000,000–5,000,000), all obtained from Sigma-Aldrich (St. Louis, MO, USA). The cells were then incubated with FITC-ECP32–41 and assayed for cell binding or internalization of ECP32–41 using cell-based ELISA or flow cytometry, respectively, as described above.

Heparinase and Chondroitinase ABC Depletion of GAGs

Beas-2B cells (3.0×10^5/well) were incubated with RPMI 1640 medium overnight in six-well plates and then treated with 5 U/ml of heparinase I, 2.4 U/ml of heparinase III, or 10 U/ml of chondroitinase ABC (Sigma-Aldrich) at 37°C for 2 h. After a PBS wash, the cells were incubated with FITC-ECP32–41 and assayed for internalization of the peptide by flow cytometry as described above.

Cell Internalization Pathway

The influence of energy on ECP32–41 internalization was measured at 4°C, 37°C, and while depleting ATP with 10 mM sodium azide and 6 mM 2-deoxy-D-glucose (Sigma-Aldrich) at 37°C for 30 min. Beas-2B cells were treated with 10 µM chlorpromazine, 5 mM methyl-β-cyclodextrin, 25 µM genistein, 2 µM filipin III, 4 µM cytochalasin D, 20 µM nocodazole, 50 mM wortmannin, or 50 µM dimethyl amiloride at 37°C for 30 min (Sigma-Aldrich). After treatment, the cells were incubated with FITC-ECP32–41 and assayed by flow cytometry as described above.

Cell Viability Assay

The effects of the peptides on cell viability were determined colourimetrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylditetrazolium bromide (MTT) (US Biological). Cells (1.0×10^4/well) were seeded into the wells of 96-well plates and incubated overnight. Cell samples were then exposed to different concentrations of TAT47–57, ECP32–41, KLA, KLA-TAT47–57 or KLA-ECP32–41. After 24 h, 100 µl of extracellular medium was added into each well, and the cells were incubated at 37°C for 3 h. The incubation medium was removed, and the remaining purple crystal formazan was dissolved in dimethyl sulfoxide. Cells treated with 0.1% Triton X-100 was used as a positive control for cell viability. A570 values were measured using a multiwall plate reader (Molecular Devices).

Membrane Disruption Assay

Lactate dehydrogenase (LDH) was used to quantify membrane disruption. The release of LDH from cells was measured by Promega CytoTox-ONE assay (Promega, USA). Cells (1.0×10^5/well) were seeded into the wells of 96-well plates and incubated overnight. Cell samples were then exposed to different concentrations of ECP32–41. After 24 h, 100 µl of extracellular medium was transferred to a black 96-well plate containing 100 µl of CytoTox-ONE reagent, incubated at RT for 10 min. Fluorescent intensity for each sample was measured using a fluorescence spectrophotometer (Wallac Victor II, Perkin Elmer, USA) and excitation and emission wavelengths of 540 nm and 590 nm respectively. LDH released from cells lysed with 0.1% Triton X-100 in medium was defined as 100% leakage and LDH released from untreated cells was set as 0% leakage.

Subcellular Fractionation

Beas-2B cells (8×10^4/dish) were cultured in 10 cm dish for 24 h, followed by incubation with 20 µM eGFP or eGFP-ECP32–41 at 4°C for 1 h. The cells were washed twice with PBS and then incubated at 37°C for 1 h, 2 h, 3 h and 4 h, separately. Cells were then homogenized and fractionated by flotation in Percoll gradients (GE Healthcare, USA) separating cytoplasm and endosomes [51]. In brief, cells were scraped off in 1 ml PBS with a rubber policeman and pelleted at 300×g for 3 min. The pellet was resuspended in 1 ml homogenization buffer (0.25 M sucrose, 3 mM Imidazole and 0.5 mM EDTA, pH 7.5) and pelleted again at 800×g for 7 min. The pellet was resuspended in 100 µl homogenization buffer with a syringe until the cells were broken but the nuclei were still intact as observed by light microscopy. The homogenate was diluted to a total volume of 1 ml with homogenization buffer. After homogenization buffer. After homogenization buffer. The gold-filled fraction was pelleted together with the nuclei at 800×g for 7 min. The pellet was resuspended in 650 µl 17% Percoll and loaded onto a 500 µl 64% sucrose cushion in a 2 ml Beckman ultracentrifuge tube. The samples were centrifuged for 90 min at 27,000×g in a Beckman SW55Ti rotor with fast acceleration to distribute the nuclear fraction at the top and the endosome-filled organelles at the bottom of the sucrose cushion. The pellet was resuspended in 100 µl homogenization buffer and referred to endosomal fraction in the results.

Western Blotting

Protein concentration from each fraction was estimated by BCA protein assay kit (Thermo). Proteins were resolved as reported in 12% SDS-PAGE and blotted to BioTrace™ polyvinylidene fluoride Membrane ( Pall Life Sciences, USA). The membrane was incubated in blocking solution (5% nonfat dry milk in PBS) for 1 h. Blots were incubated with antibodies for anti-actin (Novus Biologicals, CO), anti-lysosomal-associated membrane protein 1 (LAMP-1) (Santa Cruz Biotechnology, CA) and anti-His (Clontech Laboratories, CA) in PBS with 0.1% Tween 20 (TPBS) for 1 h. After wash with TPBS for 10 min three times, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG in TPBS at 25°C for 1 h. After wash with TPBS for 10 min three times, the protein on membrane was detected using chemiluminescent detection kit (ECL, Amersham Life Science) and chemiluminescence was measured by Kodak X-Omat film. The blotted signal was quantitated using NIH ImageJ software.

Immunohistochemical Staining

Adult female specific-pathogen-free Sprague-Dawley rats (Narl:SD) with body weights between 200 and 300 g were purchased from, and maintained at, the National Laboratory Animal Center, Taiwan. The rats were separated into two groups and injected with 5 nmol of enhanced green fluorescence protein (eGFP) or eGFP-ECP32–41 through their tail veins. All animals were asphyxiated with CO2, 1 h after injection. All major organs including brain, heart, lung, trachea, kidney, liver, spleen and intestine were removed and immediately fixed in 10% neutral-buffered formaldehyde. The tissue samples were processed by standard methods to prepare paraffin wax-embedded block samples [25]. The blocks were sectioned into 6 µm slices and were examined using a Super Sensitive Non-Biotin HRP Detection System (BioGenex Laboratories, San Ramon, CA) as previously described [25]. All these slices were then observed by using light microscope (Zeiss-Axioplan, Germany).
Statistical Analysis
Each result is reported as the mean ± standard deviation (SD), where n is the number of experiments. To compare two means, statistical analysis was performed using the unpaired Student's t-test in GraphPad Prism v4.02 (GraphPad Software, USA). One-way analysis of variance (ANOVA), followed by Dunnett’s test, was used to test for differences among multiple treatments. A P value <0.05 was considered to be statistically significant.

Supporting Information
Figure S1 eGFP-ECP32–41 in endosomal fraction. (A) Beas-2B cells were incubated with eGFP or eGFP-ECP32–41 at 4°C for 1 h. The cells were washed twice with PBS and then shifted to 37°C for further 1 h, 2 h, 3 h or 4 h. Cells were then homogenized and fractionated by flotation in Percoll gradients separating cytoplasm and endosomes. The locations of eGFP or eGFP-ECP32–41 were analysed by Western blot. (B) The blotted signal was quantitated using NIH ImageJ software and normalized to LAMP-1. The internalization of cells treated with eGFP-ECP32–41 for 2 h was set to 100%. The result is expressed as the mean ± S.D., n = 3, * P<0.05.

(TIF)

Figure S2 Cell-surface GAG-dependent cytotoxicity of KLA-ECP32–41. GAG-mediates inhibition of KLA-ECP32–41 peptide-induced cytotoxicity in Beas-2B cells. Beas-2B cells were treated with increasing concentrations of LMWH, CSC or HA for 24 h. The cytotoxicity of KLA-ECP32–41 was determined by an MTT assay. The cell viability untreated cells was set to 100%. The result is expressed as the mean ± S.D., n = 3. *,

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