Cellular Internalization of Enhanced Green Fluorescent Protein Ligated to a Human Calcitonin-Based Carrier Peptide

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Carrier peptides offer new opportunities to overcome problems in cellular drug delivery. Their objectives are improved cellular uptake or permeation of biological membranes, which are important pharmacokinetic features for the cellular distribution of therapeutics. Previously, human calcitonin (hCT) and selected C-terminal hCT fragments have been shown to be internalized and to permeate the epithelium of the nasal mucosa. To assess the potential of hCT-derived carrier peptides for cellular internalization of a model protein we fused enhanced green fluorescent protein (EGFP) and the [C^8]hCT8 – 32 fragment by using expressed protein ligation (EPL). EGFP thioester was obtained by intein-mediated purification with an affinity chitin-binding tag (the IMPACT system, based on protein splicing). Internalization of EGFP-[C^8]hCT8 – 32 by excised bovine nasal mucosa was monitored by confocal laser scanning microscopy. This novel conjugate displayed internalization into some sectors of the mucosa, whereas EGFP itself was not capable of translocation. Thus, we demonstrate successful internalization of a model protein through ligation to an hCT-derived carrier peptide, which has potential for the delivery of therapeutics. At this point the respective mechanism of translocation is unknown.

KEYWORDS:

drug delivery · enhanced green fluorescent protein · human calcitonin · membranes · protein design

Introduction

Carrier peptides that can act as shuttles for the cellular delivery of therapeutics offer new opportunities to overcome problems in drug delivery. Improved cellular uptake and membrane permeation are important pharmacokinetic features for the distribution of therapeutics.[1–3] However, peptide carriers promise to become valuable tools not only in the field of drug delivery. Carrier peptides are extremely useful for investigation of signal transduction cascades or intracellular trafficking as a result of their ability to transport large hydrophilic molecules, for example, peptides or proteins, across cellular membranes with little or no cytotoxicity. Thus the systematic investigation of carrier peptides represents a highly promising approach for the delivery of peptide, protein, and nucleic acid therapeutics. Cell-penetrating peptides such as transportan,[4] penetratins,[5] Tat-derived peptides,[6] or chimeric peptides have been reported to internalize various covalently linked cargoes.[7–9] So far the mechanisms of the internalization of cargo/carrier peptide conjugates are largely unknown and controversial. Whereas some research groups favor an absorptive endocytotic pathway for Tat-derived peptides,[10] recent experimental evidence does not support this idea.[4] The initial contact of a cationic peptide with the cell membrane appears be initiated by ionic interactions with membrane heparan sulfate proteoglycans, which are negatively charged and act as receptors for extracellular Tat protein uptake.[11] Logically, this reaction can be blocked competitively by extracellular soluble heparin. Cell lines that are deficient in membrane heparan sulfate proteoglycans are selectively impaired in the internalization of recombinant Tat fused to green fluorescent protein.

Human calcitonin (hCT), a peptide hormone of 32 amino acids, is one of the most important factors involved in the maintenance of systemic calcium homeostasis and also a powerful pain reliever for sufferers of osteolytic diseases.[12, 13] Besides studies that aim to select the most effective protocol for treatment of diseases and utilization of hCT, lately the interest in hCT has particularly focused on the therapy of established osteoporosis.[14] Previously, the internalization of hCT and a selected C-terminal hCT fragment into excised nasal epithelium has been ascribed to endocytosis of a still unknown nature.[15, 16] This mechanism is currently under investigation in our laboratories

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by the use of both cell-cultured epithelial models and excised nasal epithelium and also biophysical interaction studies with model lipid membranes.

The green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria* represents a powerful reporter molecule for monitoring gene expression, protein localization, and protein–protein interaction.[17–19] Recently, Pooga et al.[4] coupled the GFP protein to transportan by means of a disulfide linkage and observed efficient delivery of this large protein into buffalo rat liver cells. Several GFP mutant variants that differ in absorption and emission spectra and quantum yield are available to date.[20, 21] The post-translational formation of the final fluorophore requires molecular oxygen but no external enzymes or cofactors.[22] Enhanced green fluorescent protein (EGFP) is a 239 amino acid red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells.

In order to identify, visualize, and characterize new and potent carrier peptides, we fused EGFP and an hCT-derived fragment by using expressed protein ligation (EPL). This new technique elegantly combines the flexibility of chemical peptide synthesis with the extended size range of recombinant DNA expression and has been used for many suitable applications.[23–26] EPL allows generation of a recombinant protein α-thioester derivative and, based on the native chemical ligation strategy, its subsequent ligation with the N-terminal cysteine residue of a synthetic peptide.[27, 28] Furthermore, different chemical probes can be included in the peptide sequence.

Intein-mediated purification with an affinity chitin-binding tag (the IMPACT system) utilizes the inducible self-cleavage activity of a protein splicing element, that is, an intein.[29, 30] The desired target protein is bacterially expressed as a fusion protein with a C-terminal intein chitin-binding domain (CBD) tag. Affinity purification performed on the chitin beads and the subsequent thiol-induced intein cleavage result in thioester formation.[31]

We optimized the expression of the EGFP intein chitin-binding domain fusion protein in *Escherischia coli*. The isolation and purification of the EGFP reactive thioester was carried out with all steps conveniently monitored by native green fluorescence. Ligation with the human calcitonin [C^8]hCT8–32 peptide amide yielded the 265 amino acid construct. Internalization studies on bovine nasal mucosa revealed a ligation product internalized into sectors of the mucosa, as monitored by confocal laser scanning microscopy (CLSM).

Results and Discussion

Strategy

Calcitonin-based carrier peptides have been recently identified as a suitable drug translocation system. The cellular uptake of hCT was suggested to be associated with a β-sheet-induced self-assembly of the C-terminal part of the peptide. Furthermore, structure–activity studies showed that C-terminal amidation plays an essential role in uptake, which suggests the involvement of a transporter-like protein.[15, 16] In order to investigate the potential of hCT fragments for protein drug delivery, we replaced the previously reported fluorophore with enhanced green fluorescence protein (EGFP). Owing to the relevance of the peptide amide, a different strategy also had to be applied. We used expressed protein ligation as a suitable method to generate a construct of recombinant EGFP and amidated hCT-derived peptide obtained by solid-phase peptide synthesis. Ligation was performed by a native chemical ligation strategy.[28] As we used EGFP, we could easily monitor the reaction procedures by observation of fluorescence emission.

Cloning, expression, and isolation of EGFP–intein–CBD fusion protein.

The EGFP gene was amplified by PCR and subsequently in-frame cloned into the vector pTXB1. The PTXB1 vector introduces the C-terminal fusion vector of the IMPACT purification system and allows the target protein to be fused to a mini-intein from the GyrA gene of *Mycobacterium xenopi* (*Mxe* intein).[32]

The two main contributing factors that impair the yield of the desired thioester are low levels of soluble expressed fusion protein and in vivo intein cleavage. We observed no significant difference between the expression by *E. coli* bacterial strains BL21(DE3) and ER2566. Inductor concentrations, temperature, and time allowed for expression were varied in order to maximize the yield of fusion protein. However, expression levels (20 mg L^{-1}) were very low and we detected fusion protein even without induction. Mathys et al. identified aspartic and glutamic acid residues as causes of in vivo intein cleavage.[31] We observed that lysine, the last amino acid of EGFP, provoked in vivo cleavage as well (Figure 1, lane 2, band B). The cleavage varied with expression conditions; up to 35% was cleaved at 37 °C and up to 25% at 30 °C, respectively. Moreover, free intein tag with CBD attached further reduced the purification capacity of the chitin beads. In order to prevent the increased in vivo cleavage seen at higher temperature, expression of the fusion protein was performed at 15 °C overnight.

Isolation of the EGFP thioester

Chitin beads provide an optimal affinity matrix for the isolation of target proteins fused to the intein–CBD tag. The extremely high affinity of CBD for the beads allows efficient recovery of the fusion protein from the crude cell extract. In addition, stringent wash conditions reduce nonspecific binding, which increases purity of the product. The immobilized protein is then induced to undergo intein-mediated self-cleavage in the presence of thiol. The intein tag remains bound to the chitin and generated thioester is eluted.

The EGFP–intein–CBD protein maintained its green fluorescence when loaded onto the chitin column. The conditions for EGFP thioester production were optimized. Intein cleavage is influenced by the size of the column, the dilution of the cell extract prior to loading, the thiol reagent, the pH value of the cleavage buffer, the temperature, and the duration of reaction. Specific protein properties like the structure or sequence at the C-terminus of the target protein may also affect the cleavage efficiency.[33] Interestingly, none of the tested cleavage condi-

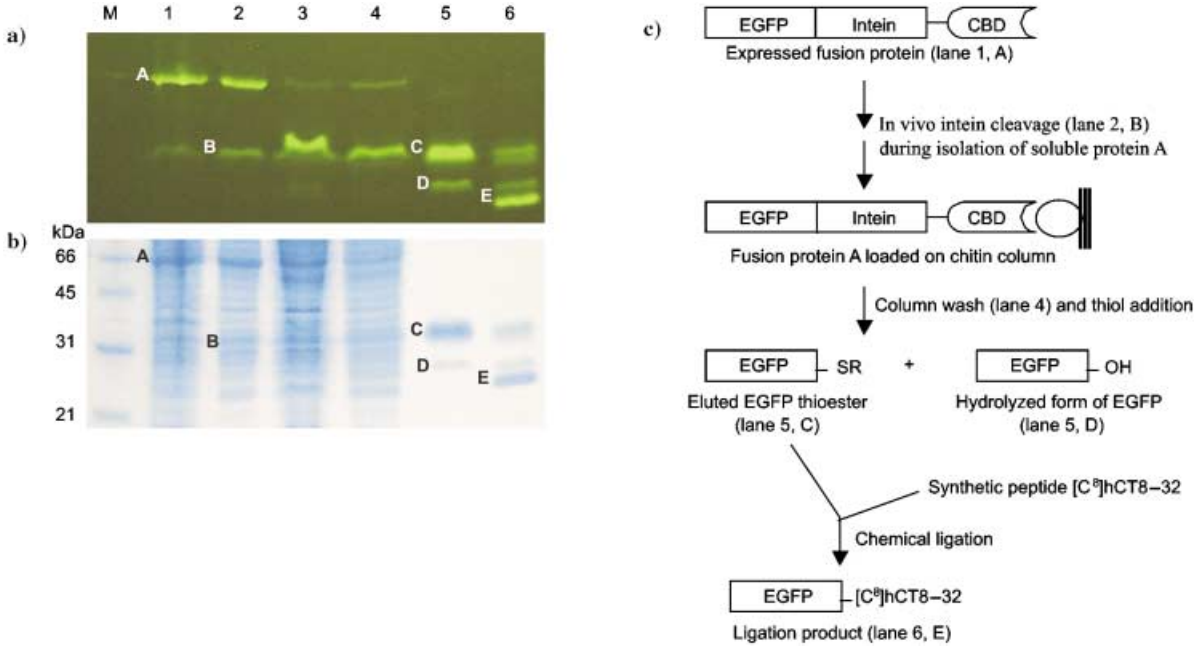

Figure 1. *Analyses of EGFP – [C⁸]hCT8 – 32 by SDS-PAGE and a schematic view of its synthesis. a) Samples without heat treatment visualized by UV irradiation at 312 nm; b) samples with (lanes 1 – 4) and without (lanes 5 – 6) heat treatment identified after staining with GelCode Blue reagent; c) scheme of EGFP – [C⁸]hCT8 – 32 synthesis: isolation and purification of EGFP – intein – CBD fusion protein, lanes 1 – 4 in (a) and (b); thioester elution and ligation with [C⁸]hCT 8 – 32 fragment, lanes 5 and 6. Lane M represents a protein marker; lane 1, crude extract after cell lysis; lane 2, supernatant after lysis; lane 3, column flow-through; lane 4, column wash; lane 5, eluted thioester; lane 6, crude ligation mixture. The in vivo cleavage product (band B) of the fusion protein (band A) remained in the column flow-through. Eluted thioester (band C), of which 10% was in the hydrolyzed form (band D), was used for ligation. The ligation product is shown as band E.*

tions had any effect on the binding of the CBD. Basic pH values favored thiol-induced cleavage but also increased the rate of thioester hydrolysis.[33, 34] Thiophenol has been shown to generate sufficient intein cleavage,[23] however, loss of green fluorescence indicated denaturation of the native EGFP structure. In contrast, 2-mercaptoethanesulfonic acid (MESNA) revealed high efficiency of intein cleavage and low thioester hydrolysis. Additionally, MESNA can be directly applied as a cofactor for expressed protein ligation.[23, 35] We obtained more than 90% intein cleavage after 25 hours at 4 °C. Cleavage progress was monitored directly on the chitin column. More than 90% pure thioester was isolated as suggested by SDS-PAGE (Figure 1, lane 5, band C) and confirmed by MALDI mass spectrometry (Figure 2 A). In order to prevent thioester hydrolysis and identify optimal storage conditions, stability tests were performed. As evident from Figure 3, the pH value of the storage buffer significantly influenced the rate of hydrolysis. Based on these observations, a pH value of 7 and low temperature (− 25 °C) were chosen for thioester storage.

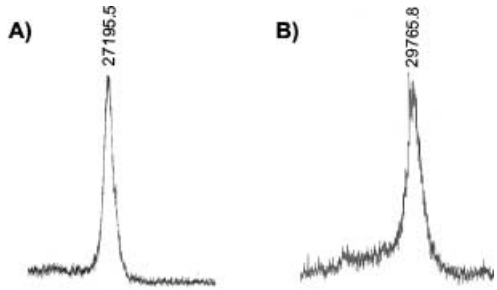

Figure 2. *Identification of EGFP thioester and EGFP – [C⁸]hCT8 – 32 by MALDI mass spectrometry. Spectra were measured at 50 μM protein concentration. A) EGFP thioester, theoretical mass 27 195.1 Da; B) ligated EGFP – [C⁸]hCT8 – 32, theoretical mass 29 765.0 Da.*

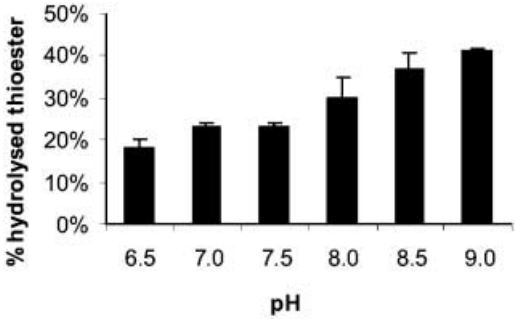

Figure 3. *Stability of EGFP thioester. The hydrolysis rate of the eluted thioester was investigated at 4 °C after 48 hours at different pH values. The percentage of hydrolyzed thioester was estimated by SDS-PAGE.*

Ligation reaction of EGFP thioester with [C⁸]hCT8 – 32

The structural domains of human calcitonin responsible for internalization have already been identified.[16] In order to perform the ligation with EGFP thioester, a cysteine residue was introduced at the N-terminus of hCT9 – 32. The modified peptide amide was obtained by solid phase synthesis. Ligations were carried out with both reactants in the millimolar concentration range and 100 mM MESNA at pH 7 and room temper

ature.The progress of the ligation was monitored and analyzed by SDS-PAGE (Figure 1, lane 6, band E) and the product was identified by MALDI mass spectrometry (Figure 2B). Further optimization studies, which included time dependency and the effect of the molar excess of peptide relative to thioester, are summarized in Figure 4. We determined 60–70% ligation efficiency at five- to tenfold molar excess of the peptide after 35 hours.

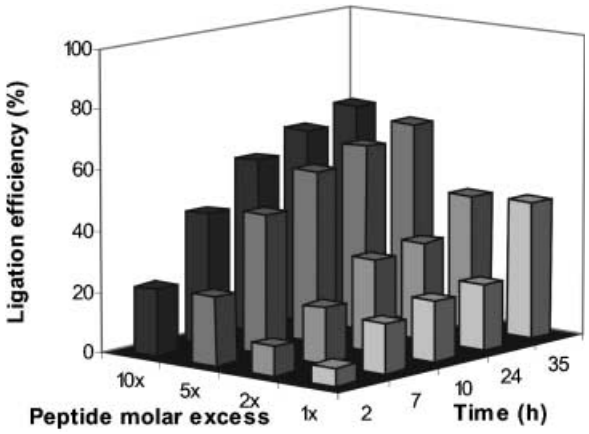

Figure 4. *Optimization of ligation efficiency. Ligation was carried out at pH 7 and room temperature. MESNA was used as a thiol cofactor.*

Electrophoretic analysis

All isolation steps and ligation reactions could be easily detected because of the fluorescence of EGFP. In all our experiments we noticed unique migration behavior of the EGFP fusion protein, thioester, and ligation product. Significant differences between the theoretical and experimental molecular masses of heat-denaturated and nondenaturated proteins were evident. Fusion EGFP–intein–CBD protein migrated at 43 kDa and retained its fluorescence when not heated. However, the heat-denaturated protein was nonfluorescent and migrated at 54 kDa, which corresponds to the theoretical value. A similar migration pattern was also noticed for the ligated EGFP–[C^8]hCT8–32 construct whereas nonfused EGFP thioester showed inverse behavior. Migration of hydrolyzed thioester was not influenced by heat treatment. The identity of all proteins could be confirmed by Western blot analysis with anti-GFP antibodies. The mechanism of this mobility shift remains unclear but this phenomenon has already been observed.[36]

Cellular internalization in excised bovine nasal mucosa as monitored by CLSM

Cell culture systems and excised nasal mucosa represent relevant in vitro models for the permeation of therapeutics across biological membranes. In order to characterize the cellular uptake of the here introduced EGFP–[C^8]hCT8–32 construct, internalization experiments with freshly excised bovine nasal mucosa were performed. The pH value of the nasal mucosa was in the range 5.5–6.5 whereas most previous studies with excised nasal tissues were performed in the pH 6–8 range.[37–39] Interestingly, in contrast to previous studies performed with hCT and fluorophore-labeled C-terminal fragments (carboxyfluorescein labels),[15, 16] we could not observe significant internalization at pH 7.4. However, experiments at the reduced pH value of 6.4 (that is, close to the physiological pH value of nasal mucosa) showed internalization of the EGFP-ligated construct and the nonligated carrier peptide into some sectors of the mucosa. At this point in our studies there is no mechanistic explanation for this observation. A concentration of 50 μM of the peptide was sufficient for internalization (Figure 5A). In contrast to the low

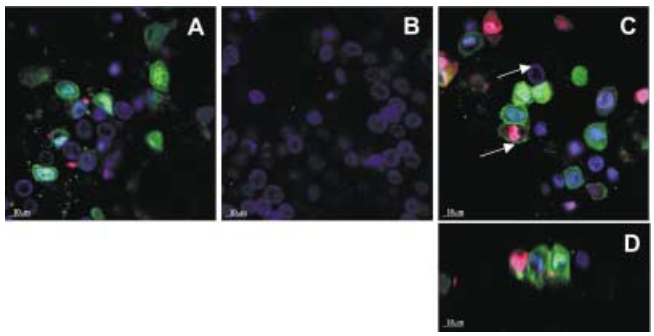

Figure 5. *Internalization as monitored by confocal laser scanning microscopy. A) Internalized [C^8(Fl)]hCT8–32 (green), Hoechst 33342 and EthD-1 staining; B) nonligated EGFP thioester (green; none visible), Hoechst 33342 and EthD-1; C) internalized EGFP–[C^8]hCT8–32 (green), Hoechst 33342 and EthD-1 nuclei coloring. D) z-scan of (C) as indicated by the two arrowheads in (C). Samples of labeled peptide (50 μM), EGFP thioester (40 μM) and EGFP–[C^8]hCT8–32 (40 μM) were dissolved in D-PBS (pH 6.4) and internalization was visualized from the mucosal side of the sample after 45 min incubation. Hoechst 33342 dye was used to stain cell nuclei (blue) and ethidium homodimer (EthD-1; red nuclei of dead cells) or calcein (green fluorescence in viable cells) were applied.*

concentrations sufficient for cellular delivery mediated by cationic carrier peptides, for example, Tat and Antennapedia-derived peptides, the higher concentrations necessary for hCT-derived peptides may represent a distinct advantage. Whereas internalized cationic peptide conjugates may further partition into deeper tissue and into the systemic circulation,[40] the therapeutics delivered by hCT-derived peptides are expected to remain in place as soon as dilution below a certain threshold level has occurred and thus exclusive delivery to the site of application is maintained. Therefore, for site-specific drug delivery the use of hCT-derived peptides would represent a strategic advantage and limit the systemic toxicity of the carrier peptide. Ubiquitous delivery is a typical feature of Tat-peptide derived conjugates[40] but may be a disadvantage when localized delivery across biologic barriers is necessary, particularly to limit systemic side effects and strictly localize drug activity.

In order to prove our concept of hCT-derived carrier peptides with potential to translocate proteins across biological membranes, we used the natural fluorescence of the EGFP–[C^8]hCT8–32 construct. As a negative control, EGFP thioester was applied, for which no internalization was detectable (Figure 5B). Figures 5C and 5D demonstrate the sectorial internalization of the construct. The EGFP–[C^8]hCT8–32 conjugate was found to be internalized into about 5–10% of the living cells of the tissue, which is the same range as that observed with the nonligated carrier peptide [C^8(Fl)]hCT8–32 alone as a positive

control (Figure 5 A). For data intepretation full preservation of the viability of the mucosa under the experimental conditions was assumed. However, toxicity of GFP might be a cause of cell death that reduces the number of vital cells.

So far demonstration of the membrane translocation potential of human calcitonin-derived carrier peptides has been limited to low molecular weight cargoes such as a carboxyfluorescein label[13]. The cellular mechanisms of the involved processes are still unknown. Initial data from our current mechanistic investigation in Madine Darby canine kidney (MDCK) cell monolayers as well as in other cell lines demonstrated that the efficiency of the translocation of fluorescence-labeled C-terminal hCT fragments decreased in the order hCT(9 – 32) > hCT(12 – 32) > hCT(15 – 32) > hCT(18 – 32). hCT(18 – 32) was the shortest translocated fragment and further truncated peptides were not internalized by MDCK cells. A vesicular-type punctiform signal pattern, already observed in nasal epithelium for hCT and hCT(9 – 32), was also observed in MDCK. This punctiform pattern combined with the absence of significant uptake at 4 °C suggests endocytosis as the mechanism of uptake.

Here, we present for the first time the possibility of use of hCT-derived carrier peptide to translocate a larger molecular weight cargo, that is, an EGFP fusion protein construct, across a biological membrane. We showed that neither the additional cysteine spacer nor the N-terminal extension reduces the internalization capacity of the hCT fragments. Application of expressed chemical ligation to recombinant EGFP was found to be an elegant procedure to monitor and optimize the technique. This novel EGFP conjugate revealed specific mucosal internalization, whereas EGFP alone was not capable of permeation. Several proteins have been reported to be transported into cells with the help of carrier peptides, both functional proteins[40, 41] and GFP.[42] GFP has to be folded correctly for fluorescence emission upon irradiation. The research group that first reported its use as a fusion protein with the herpes virus protein VP22 had to use indirect immunodetection since intrinsic fluorescence could not be determined.[42] Another group who used the VP22 system did not observe delivery of intracellular GFP fluorescence at all.[43] Pooga et al. reported the use of GFP coupled to transportan by a disulfide linkage.[4] After fixation and permeabilization of the cells, intracellular fluorescence was detected. We report here direct detection of intracellular fluorescence of GFP in living, unfixed, and unpermeabilized cells, with the carrier peptide linked to its cargo by native chemical ligation. The result is a stable peptide bond, not prone to reductive cleavage. Accordingly, the hCT system offers new opportunities for the delivery of therapeutics across cellular membranes that may be further extended to larger proteins, nucleic acids, and hydrophilic compounds. In contrast to other cell-penetrating peptides, interaction with the uncharged C-terminal domain of hCT-derived carrier peptides does not require ionic interaction. This lack of charge avoids the potential toxicity of highly positively charged carrier entities typical for poly(lysine) and poly(arginine)[44] and also expected for Tat-derived peptides when in contact with the negatively charged extracellular matrix that coats the cellular membrane. The neurotoxicity as well as the cytoxicity of Tat towards lymphocytes have been demonstrated in the literature.[45–47] On the other hand, calcitonins have a long-term safety record based on their widespread use as a nasally administered therapeutic, for example, to treat osteoporosis. At this point, interpretations regarding the cellular mechanism of hCT-derived carrier peptides remain elusive. However, their mechanism is expected to differ markedly from those of the highly cationic carrier peptides. Thus, hCT-derived peptides could substantially supplement the biochemical tools necessary to overcome cellular barriers for efficient drug delivery.

Experimental Section

DNA constructs: The DNA that encodes EGFP (239 amino acid residues) was amplified by PCR from the pEGFP-N1 vector (Clontech) by using the EGFP forward primer 5′-GGTGGTCATATGATGGTGAG-CAAGGGCGAGG-3′ and EGFP reverse primer 5′-GGTGGTTGCTCTT-CCGCACTTGTACAGCTCGTCCATGC-3′ (MWG-Biotech AG). Primers were designed to introduce the *Nde*I and *Sap*I sites in the forward and reverse primers, respectively. After digestion and purification, the PCR fragment was inserted into *Nde*I-*Sap*I-treated C-terminal fusion vector pTXB1 (New England Biolabs). DNA sequencing was used to confirm in-frame cloning of the EGFP gene.

Fusion protein expression in *E. coli*: *E. coli* BL21(DE3) or ER2566 cells transformed with pTXB1-EGFP plasmid were grown in LB medium that contained 100 μg ml^{-1} ampicillin until they reached the mid-log phase and were induced with isopropylthiogalactosid (0.3 mM). Cells were harvested by centrifugation after overnight expression at 15 °C. Lysis of the cells was performed over 1 hour at 4 °C in buffer A (tris(hydroxymethyl)aminomethane (Tris) – HCl (20 mM), ethylenediaminetetraacetate (EDTA; 1 mM), NaCl (500 mM); pH 8) in the presence of Triton X-100 (0.7 %), phenylmethanesulfonyl fluoride (20 μM), tris(carboxyethyl)phosphine (0.7 mM), and lysozyme (15 μg ml^{-1}). Three to four lysis extraction rounds and sonication were necessary to complete the lysis. The soluble protein extract was isolated by centrifugation. Expression and isolation of the target protein was monitored by SDS-PAGE. Modified sample buffer without dithiothreitol, which induces intein cleavage, was used for gel analysis. Semidry Western blot in the presence of 1:5000 anti-CBD serum (New England Biolabs) or 1:300 polyclonal GFP antibody (Clontech) confirmed the expression of EGFP – intein – CBD fusion protein. Samples without prior heat treatment were directly visualized on the gel by UV irradiation (312 nm).

Purification and isolation of EGFP thioester: A column (Econo Column, Biorad) filled with chitin beads was equilibrated at room temperature with 10 bed volumes of buffer A. The extract that contained the fusion protein was loaded at 4 °C onto the column at a flow rate of 0.5 mL min^{-1}. Samples from the flow-through analyzed by SDS-PAGE indicated a high binding efficiency of the fusion precursor on the chitin. Washing with 10 bed volumes of buffer A reduced nonspecific binding of other proteins. Intein cleavage was induced by rapid flushing with 3 bed volumes of buffer A that contained the sodium salt of MESNA (100 mM). On-column cleavage proceeded at 4 °C for 24 – 40 hours. Cleavage efficiency was monitored by SDS-PAGE analysis of induced resin slurry (100 μL) mixed with sample buffer (30 μL). The supernatant was analyzed by SDS-PAGE and visualized by UV irradiation prior to staining with GelCode blue staining reagent (Pierce). EGFP thioester was eluted at room temperature with elution buffer (Tris – HCl (20 mM), EDTA (1 mM), NaCl (50 mM); pH 7) in 1 mL fractions and the protein content was determined by Bradford assay. The thioester identity was confirmed by MALDI mass spectrometry.

Synthesis of hCT-derived carrier peptides: The hCT fragments were synthesized by the Fmoc/*t*Bu (fluorenylmethoxycarbonyl/*tert*-butyl) solid-phase strategy with an automated multiple peptide synthesizer (Syro, MultiSynTech),[48] and labeled with 5(4)-carboxyfluorescein (Fl) at the N terminus.[49] Analysis was carried out by MALDI mass spectrometry (Voyager II, Perseptive) and reversed-phase HPLC on a C18 column with an acetonitrile/water (0.1% trifluoroacetic acid) gradient. [C^8]hCT8–32, CLGTYTQDFNKFHTFPQTAIGVGAP-NH_2, MALDI: calcd.: 2712.8; found: 2713.7; retention time: 14.7 min with 10–60% acetonitrile in 30 min. [C^8(Fl)]hCT8–32, (Fl)-CLGTYTQDFNKFHTFPQTAIGVGAP-NH_2, MALDI: calcd.: 3070.4; found: 3070.2; retention time: 17.5 min with 10–70% acetonitrile in 30 min.

Ligation of the EGFP thioester with [C^8]hCT8–32: Ligation reactions were carried out with thioester (0.1 mM) in Tris–HCl (5 mM; pH 7) at room temperature in the presence of MESNA (100 mM). The lyophilized synthetic peptide was dissolved in the reaction buffer in different amounts of molar excess compared to the thioester. Progress of the ligation was monitored by SDS-PAGE and subsequent UV detection, and the expected mass was confirmed by MALDI mass spectrometry.

Internalization studies on bovine nasal mucosa: Bovine nasal mucosa was obtained from freshly slaughtered cattle at the slaughterhouse (Schlachthaus AG, Zürich, Switzerland) and prepared as described previously.[50] After 20 min equilibration of the excised mucosa in Dulbecco's modified phosphate-buffered saline (D-PBS) at 37 °C, the incubation solution was replaced by carrier peptide solution (50 μM), thioester (40 μM), or ligation product dissolved in D-PBS (pH 6.4, final volume 200 μL) and this solution was then incubated for 45 min at 37 °C.

Visualization by confocal laser scanning microscopy: Cells were washed with D-PBS (pH 6.4) then internalization was monitored by CLSM. Viability was tested by using a staining assay (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes Europe, Leiden, The Netherlands). EthD-1 (8 μM final concentration) was applied for 30 min at 37 °C during or after sample incubation. Unfixed cell nuclei were stained over 30 min at 37 °C with Hoechst 33342 (Molecular Probes Europe) at a final concentration of 2 μg ml^{-1}. A Zeiss LSM 410 inverted microscope (Zeiss AG, Zurich, Switzerland) was used for CLSM. Optical sections were taken with a 63 × /1.2 objective. Image processing was carried out on a Silicone Graphics workstation (Zurich, Switzerland) by using IMARIS software (Bitplane AG, Zurich, Switzerland).

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