Decoding the Entry of Two Novel Cell-Penetrating Peptides in HeLa Cells: Lipid Raft-Mediated Endocytosis and Endosomal Escape†

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ABSTRACT: Cellular entry of peptide, protein, and nucleic acid biopharmaceuticals is severely impeded by the cell membrane. Linkage or assembly of such agents and cell-penetrating peptides (CPP) with the ability to cross cellular membranes has opened a new horizon in biomedical research. Nevertheless, the uptake mechanisms of most CPP have been controversially discussed and are poorly understood. We present data on two recently developed oligocationic CPP, the sweet arrow peptide SAP, a γ-zein-related sequence, and a branched human calcitonin derived peptide, hCT(9−32)-br, carrying a simian virus derived nuclear localization sequence in the side chain. Uptake in HeLa cells and intracellular trafficking of N-terminally carboxyfluorescein labeled peptides was studied by confocal laser scanning microscopy and flow cytometry using biochemical markers in combination with quenching and colocalization approaches. Both peptides were readily internalized by HeLa cells through interaction with the extracellular matrix followed by lipid raft-mediated endocytosis as confirmed by reduced uptake at lower temperature, in the presence of endocytosis inhibitors and through cholesterol depletion by methyl-β-cyclodextrin, supported by colocalization with markers for clathrin-independent pathways. In contrast to the oligocationic SAP and hCT(9−32)-br, interaction with the extracellular matrix, however, was no prerequisite for the observed lipid raft-mediated uptake of the weakly cationic, unbranched hCT(9−32). Transient involvement of endosomes in intracellular trafficking of SAP and hCT(9−32)-br prior to endosomal escape of both peptides was revealed by colocalization and pulse−chase studies of the peptides with the early endosome antigen 1. The results bear potential for CPP as tools for intracellular drug delivery.

On the basis of the largely enhanced discovery process toward novel pharmacologically active agents, increasing numbers of potential peptide, protein, or nucleic acid based biopharmaceuticals are considered for therapeutic application and drug development. Nevertheless, due to their large molecular size, charge, and polarity, the clinical development of these biomacromolecules is likely to be problematic because of their insufficient ability to cross cellular membranes or reach intracellular targets. This explains their often poor or zero bioavailability and clinical efficacy. The discovery of various 10−30-mer peptides with the ability to translocate cellular membranes has, therefore, opened a new horizon in biomedical research (*1*). Chemical ligation or physical assembly of these so-called cell-penetrating peptides (CPP)[1] with biopharmaceuticals of poor cellular access is currently a main avenue in engineering delivery systems that could mediate the noninvasive import of such problematic cargoes into cells. Various oligocationic cell-penetrating peptides, e.g., penetratin, the Antennapedia homeodomain derived CPP from *Drosophila* (*2*), HIV-1-derived Tat peptides (*3*), or oligoarginine peptides (*4*), have been well described in the literature. They were widely considered for the therapeutic delivery of peptides (*5*), proteins (*6*), oligonucleotides (*7*), plasmids (*8*), peptide−nucleic acids (PNAs) (*9*), and even nanoparticles (*10*). Besides these oligocationic CPP, enhanced translocation of the cellular membrane has also been reported for weakly cationic peptides, e.g., hCT(9−32), a human calcitonin-derived CPP that has been introduced by our group (*11*, *12*). Despite the widespread interest in such molecular carriers, the mechanisms underly-

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[1] Abbreviations: BSA, bovine serum albumin; CF, carboxyfluorescein; CLSM, confocal laser scanning microscopy; CPP, cell-penetrating peptide(s); DMEM, Dulbecco's modified Eagle's medium; DOG, 2-deoxyglucose; ECM, extracellular matrix; EEA1, early endosome antigen 1; FACS, fluorescence-activated cell sorting; Gal T, galactosyltransferase; HBSS, Hank's balanced salt solution; hCT, human calcitonin; HIV-1, human immunodeficiency virus 1; HP-γ-CD, hydroxypropyl-γ-cyclodextrin; M-β-CD, methyl-β-cyclodextrin; NaN_3, sodium azide; NLS, nuclear localization sequence; PNA, peptide−nucleic acid; SAP, sweet arrow peptide; SV40, simian virus 40.

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Table 1: Name, Sequence, and Origin of the Cell-Penetrating Peptides (CPP) Used[a]

Name	Sequence	Origin
hCT(9-32)	LGTYTQDFNKFHTFPQTAIGVGAP-NH_2	human calcitonin
hCT(9-32)-br	LGTYTQDFNKFHTFPQTAIGVGAP-NH_2 | AFGVGPDEVKRKKKP–NH_2	human calcitonin, simian virus 40
SAP	VRLPPP-VRLPPP-VRLPPP	modified maize zein sequence

[a] Throughout, peptides were CF-labeled at the N-terminus.

ing the cellular translocation of CPP are yet incompletely understood and were subject to controversial discussions. For instance, the cellular translocation of penetratin and Tat peptides was initially assigned to a passive, temperature-independent process (*2*, *3*, *13*), not sensitive to endocytosis inhibitors (*3*, *14*). The observations were thought to be consistent with a theoretical model for a CPP-induced physical perturbation of the lipid membrane leading to a direct translocation of the plasma membrane (*2*, *3*, *15*). Recently, however, the related hypotheses involving direct translocation have been challenged following several reports on artifactual results that were caused by cell fixation prior to confocal laser scanning microscopy (CLSM) of cells incubated with fluorescence-labeled CPP. Another source of misinterpretation was found in the experimental difficulties to distinguish cell surface-associated CPP from CPP internalized in cytoplasmic compartments (*16*). Unequivocal discrimination between associated and internalized fluorescence, however, is a prerequisite to interpret both CLSM and fluorescence-activated cell sorting (FACS) analysis with labeled CPP. More recent work demonstrated clearly the involvement of endocytosis in the internalization of a Tat-derived peptide (*16*) and penetratin (*17*). Prior to endocytosis, both peptides were shown to first interact electrostatically with the extracellular matrix (ECM) of the cell surface through binding to negatively charged glycosaminoglycans (*18*). Endocytosis may involve several pathways. So far, most classical studies focused on the pathway via clathrin-coated pits (*19*−*20*). Meanwhile, however, technologies and reagents are available that can shed light on nonclassical, clathrin-independent pathways such as endocytosis via lipid rafts (*21*−*23*). Lipid rafts are membrane microdomains that are enriched in cholesterol and sphingolipids (*24*, *25*). The sensitivity of lipid raft-mediated endocytosis to cholesterol depletion distinguishes this pathway from non-raft-dependent processes such as clathrin-mediated endocytosis (*22*, *23*, *26*, *27*). Lipid raft pathways mediate the internalization of sphingolipid binding toxins such as cholera toxin (*22*, *23*, *26*), whereas transferrin, a typical marker for clathrin-mediated endocytosis, is excluded from lipid rafts (*24*, *25*, *28*).

Here we investigate the cellular entry of two N-terminally carboxyfluorescein- (CF-) labeled, oligocationic CPP representing more recent discoveries in the field. One is a branched derivative of the linear human calcitonin-derived CF-hCT(9−32) (*11*), denoted as CF-hCT(9−32)-br (*29*), that carries an oligocationic, SV40-derived nuclear localization sequence, the GPDEVKRKKKP motif, in the form of a side branch to the main peptide chain (see Table 1). The other one is the equally CF-labeled linear proline-rich sweet arrow peptide CF-SAP (see Table I), a linear trimer of repetitive VRLPPP domains and conceived as an amphipathic version of a polyproline sequence related to γ-zein, a storage protein of maize (*30*−*32*). As compared to other CPP, both peptides offer practical advantages including good solubility in water, low cytotoxicity, and, in case of CF-SAP, nonviral origin (*29*, *31*). As a positive control we used the unbranched CPP CF-hCT(9−32) (*11*). We report on their uptake into HeLa cells as assessed by CLSM and confirmed by flow cytometry. For their cellular entry we propose a nonclassical, clathrin-independent pathway through lipid raft-mediated endocytosis. Having passed the cellular membrane, the two cationic compounds were found to transiently sojourn in endosomal compartments where sorting steps take place. A pulse−chase study revealed the endosomal escape of the peptides and their subsequent transfer to other cytoplasmic vesicles.

EXPERIMENTAL PROCEDURES

Materials

HeLa cells were obtained from American Type Culture Collection ATTC (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide], and saponin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Trypsin−EDTA, penicillin, streptomycin, Hank's balanced salt solution (HBSS), and phosphate buffer solution (PBS, pH 7.4) without calcium and magnesium were from Life Technologies (Basel, Switzerland). Foetal calf serum (FCS) was purchased from Winiger AG (Wohlen, Switzerland). Hoechst 33342, cholera toxin subunit B (recombinant) Alexa Fluor 594 conjugate, and tetramethylrhodamine-labeled transferrin were from Molecular Probes (Leiden, The Netherlands). The monoclonal first antibody rabbit anti-EEA1 was from Affinity BioReagents (Golden, CO), the second antibodies donkey anti-rabbit biotin and donkey anti-mouse Texas red were from Jackson Immuno Research Laboratories (West Grove, PA) and streptavidin labeled with Texas red, Cy5, or FITC from Amersham Biosciences (Uppsala, Sweden). The monoclonal first antibodies mouse anti-Gal T and mouse anti-P63 were a kind gift of Dr. Jack Rohrer from the Institute of Physiology, University of Zurich (Zurich, Switzerland). Dako fluorescent mounting medium was purchased from DAKO Corp. (Carpinteria, CA). 5(6)-Carboxyfluorescein (CF), heparin, Trypan blue, sodium azide (NaN_3), Triton X-100, menadione (2-methyl-1,4-naphthoquinone), and 2-deoxyglucose were obtained from Fluka (Buchs, Switzerland), and methyl-β-cyclodextrin (M-β-CD) as well as 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) was from Wacker-Chemie (Munich, Germany). Glass chamber slides were obtained from Nunc (Wiesbaden, Germany). Cell culturing flasks (25 cm^2) and 24-well plates were from TPP (Trasadingen, Switzerland). Coverslips and microscope slides were purchased from Knittel (Braunschweig, Germany).

Methods

Peptide Synthesis. N-Terminally fluorescence-labeled sweet arrow peptide (CF-SAP) (see Table 1) was synthesized by solid-phase peptide synthesis on a 2-chlorotrityl resin following the 9-fluorenylmethoxycarbonyl/*tert*-butyl strategy

prior to fluorescent labeling with 5(6)-carboxyfluorescein (CF) (*31*); CF-hCT(9−32) and CF-hCT(9−32)-br (see Table 1) were synthesized according to the Fmoc strategy by automated multiple solid-phase peptide synthesis using a robot system (Syro, MultiSynTech, Germany). Introduction of the side chain into the branched peptide, CF-labeling, and identification were performed as described previously (*29*).

Cell Culture. HeLa cells were cultured as exponentially growing subconfluent monolayers at 37 °C under 5% CO_2. The cell culture was in 25 cm^2 culture flasks in DMEM (high glucose) supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. Medium was exchanged three times per week. Exponentially growing HeLa cells were seeded at a constant density of about 10^5 cells/cm^2 on chamber slides, 24-well plates, or coverslips. For experiments cells were used 24 h postseeding.

XTT Cell Viability Assay. To check for cell proliferation and viability of HeLa cells after treatment with hCT(9−32) or hCT(9−32)-br, we measured the overall activity of mitochondrial dehydrogenase by XTT assays (*33*). HeLa cells were grown in 96-well plates until 70−80% confluency and then incubated with peptide solutions in medium ranging from 30 to 100 μM at 37 °C for 2 h. As negative or positive controls, we used untreated cells or cells treated with 70% EtOH for 7 min, respectively. After the peptide solutions were discarded and after several washing steps, XTT solution was added for 2 h at 37 °C. The overall activity of mitochondrial dehydrogenase in each well was measured spectrophotometrically at 450 nm using a SpectraFluor Plus (Tecan, Crailsheim, Germany).

Confocal Laser Scanning Microscopy (CLSM) of CPP Uptake. For uptake, cells were equilibrated in HBSS for several minutes prior to incubation with HBSS containing either CF-SAP (50 μM), CF-hCT(9−32)-br (30 μM), CF-hCT(9−32) (30 μM), or unconjugated fluorophore (50 or 30 μM, respectively) for 1 h. After 30 min, Hoechst 33342 was added to a final concentration of 1 μg/mL for nuclear staining. The study was conducted at 37 or 4 °C under horizontal mechanical shaking at 150 min^{-1}. For inhibition of endocytosis, cells were pretreated for 1 h with NaN_3/2-deoxyglucose (0.1%/50 mM in HBSS), heparin (5 units/mL in PBS), or methyl-β-cyclodextrin (10 mM in serum free medium) prior to incubation with CPP.

Subsequently, cells were washed three times with PBS and inspected immediately in a HBSS solution without any fixation using a Zeiss CLSM 410 inverted microscope (*34*) with a 63×, 1.4 NA plan apochromatic lens (lasers: HeNe 543 nm, Ar 488 nm, and Ar UV 364 nm). Three-dimensional multichannel image processing was performed using IMARIS software (Bitplane AG, Zurich, Switzerland) on a Silicon Graphics workstation. Background fluorescence was determined by analyzing nontreated cells.

Uptake and Endocytosis Inhibition Study by FACS. HeLa cells were seeded in 24-well plates and incubated with CF-SAP (50 μM), CF-hCT(9−32) (30 μM), or CF-hCT(9−32)-br (30 μM) in HBSS for 3 h. After incubation, cells were washed three times with PBS, trypsinized for 10 min, resuspended in medium, and immediately placed on ice. To quench potentially remaining external fluorescence, Trypan blue was added to the samples before analysis by FACS on a FacScan (Becton Dickinson, Franklin Lakes, NJ) within 2 h after trypsinization. A total of 10000 cells per sample was analyzed. The mean fluorescence intensity of peptide-labeled cell populations was compared to nontreated control cells and control cells incubated with 5(6)-carboxyfluorescein.

For inhibition of endocytosis, cells were pretreated for 1 h with NaN_3/2-deoxyglucose (0.1%/50 mM in HBSS), heparin (5 units/mL in PBS), or methyl-β-cyclodextrin (10 mM in serum free medium) prior to incubation with CPP.

Colocalization Study by CLSM. HeLa cells were seeded on coverslips (12 mm) in 24-well plates as described above. After equilibration, cells were coincubated for 30 min in solutions of CF-SAP (50 μM), CF-hCT(9−32)-br, or CF-hCT(9−32) (both 30 μM) with either cholera toxin (10 μg/mL) or transferrin (50 μg/mL) in HBSS. Cells were washed three times with PBS and fixed in 1% (v/v) aqueous formaldehyde solution at room temperature for 30 min. Cells were washed again with PBS and analyzed using a high-resolution TCS-SP2 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany) with a 63×, 1.4 NA plan apochromatic lens using HeNe 594 nm, HeNe 543 nm, Ar 488 nm, and Ar UV 405 nm lasers. To avoid cross talk, emission signals were collected independently. Image processing was performed using IMARIS software (Bitplane AG, Zurich, Switzerland) on a Silicon Graphics workstation. The images were deconvolved using Huygens software (Scientific Volume Imaging B.V., Hilversum, The Netherlands).

Immunofluorescence Labeling. Tracing Early Endosomes. HeLa cells seeded on coverslips were incubated with CF-SAP or CF-hCT(9−32)-br for 30 min as described and at the same time incubated with 1 μg/mL Hoechst 33342 to stain the nuclei. To monitor the intracellular fate of the ingested CPP, pulse−chase studies (*35*) were performed with a 30 min CPP incubation period, followed by several PBS washings, and with a chase period of 2.5 h incubation in DMEM. Cells were fixed for 30 min in 1% (v/v) formaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 1 min at room temperature. Cells were washed several times with PBS. Nonspecific binding sites were blocked by incubating for 30 min with 1% BSA in PBS. Cells were then incubated with rabbit anti-EEA1 (1:250) in 1% BSA in PBS for 1 h at room temperature. After another washing step, cells were incubated with donkey anti-rabbit biotin (1:200) in 1% BSA in PBS for 1 h at room temperature. After being washed three times with PBS, streptavidin conjugated to Texas red (1:100) in 1% BSA in PBS was added to cells incubated with donkey anti-rabbit biotin for 1 h at room temperature. All samples were washed again three times with PBS and mounted in Dako mounting medium.

Immunofluorescence Labeling. Tracing the Endoplasmic Reticulum and the Golgi Network. HeLa cells seeded on coverslips were incubated with CF-SAP or CF-hCT(9−32)-br for 30 min as described and simultaneously incubated with 1 μg/mL Hoechst 33342 to stain the nuclei. Cells were fixed for 30 min in 1% (v/v) formaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 1 min at room temperature. Cells were washed several times with PBS. Nonspecific binding sites were blocked by incubating for 30 min with 1% BSA in PBS. Cells were then incubated with mouse anti-Gal T (1:2) or mouse anti-P63 (1:200) in 1% BSA in PBS for 1 h at room temperature. After another washing step, cells were incubated with donkey anti-mouse Texas red (1:200) in 1% BSA in PBS for 1 h at room temperature. All

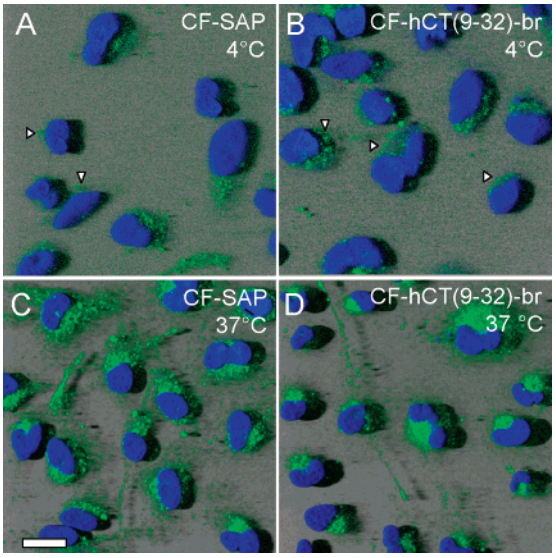

FIGURE 1: Cellular uptake of CF-SAP and CF-hCT(9−32)-br in HeLa cells at different incubation temperatures. HeLa cells were incubated for 1 h with 50 μM CF-SAP (A, C) or 30 μM CF-hCT-(9−32)-br (B, D). Upper panels (A, B): incubation at 4 °C. Lower panels (C, D): incubation at 37 °C. Arrowheads indicate accumulation of CPP on cellular membranes when incubation was performed at 4 °C. The scale bar is 30 μm. This is a 3D representation.

samples were washed again three times with PBS and mounted in Dako mounting medium.

Cells were scanned as described for colocalization studies. As control experiments, cells incubated with the secondary antibodies without primary antibody staining confirmed the high specificity of the antibodies.

RESULTS

Cellular Uptake in HeLa Cells. As visualized by CLSM, after incubation at 37 °C, HeLa cells showed marked cellular uptake when exposed to either one of the two cationic peptides, CF-SAP and CF-hCT(9−32)-br, confirming both peptides as potential cell-penetrating peptides (panels C and D of Figure 1, respectively). The cellular uptake of CF-hCT-(9−32) as control peptide was also demonstrated (data not shown). The punctuated fluorescence pattern indicated that the translocated fluorescence was localized in discrete vesicular compartments in the cytoplasm, suggestive of an endocytic pathway of internalization. This conclusion was supported by the observation that the internalization was a temperature-dependent process as cellular uptake did not take place at 4 °C for both CF-SAP and CF-hCT(9−32)-br, respectively (panels A and B of Figure 1, respectively). Some accumulation of the peptides in the periphery of cells was also observed (Figure 1, arrowheads), supporting the assumption of cellular binding between the cationic peptides and the negatively charged ECM of HeLa cells prior to uptake. Nontreated cells and cells incubated with the fluorescence marker carboxyfluorescein alone were analyzed as controls. No intracellular fluorescence was observed in both cases (data not shown).

Quantitative Assessment of Peptide Internalization by FACS Analysis. For confirmation of these findings we quantified the internalization of the two peptides by FACS analysis. Panels A and B of Figure 2 show HeLa cells after incubation with CF-hCT(9−32)-br and CF-SAP, respectively,

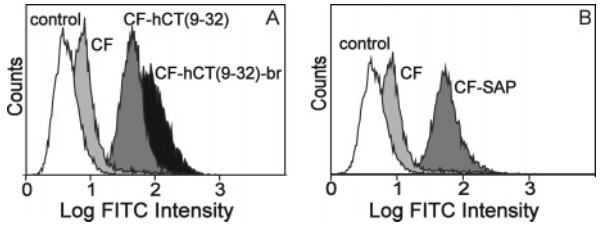

FIGURE 2: Uptake of CPP as determined by FACS analysis. (A, B) Frequency distributions of fluorescence intensity in HeLa cells incubated for 3 h with 30 μM CF-hCT(9−32), 30 μM CF-hCT-(9−32)-br (A), or 50 μM CF-SAP (B), at 37 °C. Open distributions represent nontreated control cells, light gray distributions stand for cells treated with the fluorescence marker 5(6)-carboxyfluorescein (CF), dark gray distributions show HeLa cells incubated with CF-SAP or CF-hCT(9−32), and the black distribution in panel A represents CF-labeled CF-hCT(9−32)-br.

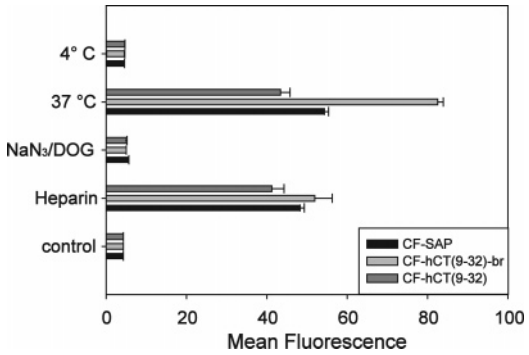

FIGURE 3: Uptake and endocytosis inhibition of CF-SAP, CF-hCT-(9−32)-br, and CF-hCT(9−32) in HeLa cells as determined by FACS analysis. HeLa cells were incubated for 3 h at either 37 or 4 °C with 50 μM CF-SAP (black bars), 30 μM CF-hCT(9−32)-br (light gray bars), or 30 μM CF-hCT(9−32) (dark gray bars). Endocytosis inhibition was performed by pretreating cells with 0.1% NaN_3/50 mM 2-deoxyglucose (NaN_3/DOG) or 5 units/mL heparin before incubation with CF-SAP, CF-hCT(9−32)-br, or CF-hCT-(9−32). Nontreated cells were used as control cells.

which resulted in uptake and labeling of the cells with both peptides. Nontreated cells and cells incubated with free carboxyfluorescein were used as negative controls. As positive control, the nonbranched CF-hCT(9−32) was used. As shown in Figure 2A, internalization of CF-hCT(9−32)-br was significantly more efficient than CF-hCT(9−32). It is likely that the enhancement resulted from the oligocationic NLS sequence in the side chain of CF-hCT(9−32)-br (see Table 1).

Uptake Studies under Endocytosis Inhibition. Next we investigated the internalization of CF-SAP, CF-hCT(9−32)-br, and the control peptide CF-hCT(9−32) under the influence of various endocytosis inhibiting conditions. Again, internalization was quantified by FACS analysis. As negative controls, nontreated cells and cells incubated with carboxyfluorescein alone (data not shown) were analyzed. As expected, uptake of CF-SAP and CF-hCT(9−32)-br as well as CF-hCT(9−32) was largely reduced when incubation was performed at 4 °C (Figure 3). Previously, inhibition of endocytosis at 4 °C was attributed not only to a blockade of active processes but also to the rigidification of the lipid membrane at this temperature (*36*). Therefore, we also tested peptide internalization at 37 °C after preincubation with NaN_3/2-deoxyglucose, which was expected to impair energy-dependent translocation by ATP depletion. As shown in Figure 3, internalization of all three peptides was blocked

through pretreatment with NaN_3/2-deoxyglucose, again suggesting endocytic internalization as the underlying translocation mechanism.

It was previously demonstrated that the endocytic translocation of the Tat peptide is triggered by its electrostatic interaction with the negatively charged sulfated proteoglycans of the ECM, as suggested by an inhibitory effect of heparin (*37*). Therefore, we examined the possibility that ECM-based sulfated proteoglycans contribute to the endocytosis of CF-SAP and CF-hCT(9−32)-br. Again, the nonbranched CF-hCT(9−32) was used as a control peptide. A marked drop in internalization by about one-third was observed with CF-hCT(9−32)-br only. In contrast, the drop was minor with CF-SAP and absent with CF-hCT(9−32). The difference is likely to result from the high charge density in the cationic NLS domain in the side chain of CF-hCT(9−32)-br versus only one cationic amino acid per VRLPPP unit for the CF-SAP trimer molecule. For the weakly cationic CPP CF-hCT-(9−32) carrying one positive charge only no suppression was seen after heparin treatment. The result confirmed the role of sulfated proteoglycans in binding oligocationic CPP to the ECM and in assisting in their internalization. Nevertheless, interaction with sulfated proteoglycans alone did not appear to be an exclusive prerequisite for the internalization of the oligocationic peptides, CF-SAP and CF-hCT(9−32)-br, since the weakly cationic CPP CF-hCT(9−32) was also internalized in HeLa cells. The results indicate contrasting features of internalization between oligocationic and weakly cationic peptides. All endocytosis inhibition studies were also performed qualitatively by CLSM, corroborating the above decribed results (data not shown).

No Endocytosis via Clathrin-Coated Endosomes. We investigated the endocytic pathway involved in the internalization process of both CF-SAP and CF-hCT(9−32)-br. Transferrin is a well-known marker for its internalization via clathrin-coated pits on the plasma membrane that eventually invaginate and detach from the membrane to form clathrin-coated vesicles. The cargo in those clathrin-coated vesicles undergoes further trafficking to endosomes where sorting to lysosomes may occur (*35*, *38*). To assess whether this scenario could be the case for the two novel peptides, we performed a colocalization study. CF-SAP and CF-hCT-(9−32)-br, respectively, were each coincubated together with TRITC-labeled transferrin in HeLa cells. Figure 4B shows CLSM micrographs of an overlay of CF-SAP and transferrin, together with related panels of the individual contributions of the peptides and transferrin alone. Predominantly distinct internalization patterns of the peptides and the marker with only minor colocalization were observed that point toward an essentially clathrin-independent endocytosis. A corresponding set of experiments was also performed with CF-hCT(9−32)-br and transferrin. Again, the obtained vesicular patterns of both components were mostly distinct from each other with only few colocalized vesicles of CF-hCT(9−32)-br and transferrin (Figure 4D). This result excludes a major role of clathrin-mediated endocytosis for the cellular translocation of CF-SAP and CF-hCT(9−32)-br and suggests clathrin-independent uptake mechanisms for these CPP.

Endocytosis Occurs via Lipid Rafts. The involvement of clathrin-coated endosomes in the internalization process of CF-SAP and CF-hCT(9−32)-br was further ruled out by the assessment that both peptides colocalize with cholera toxin. Similar to other microbial toxins, the active fraction of cholera toxin is internalized by a clathrin-independent endocytosis involving lipid rafts (*22*, *23*, *26*). Lipid rafts are cell membrane microdomains enriched in cholesterol and sphingolipids. For this assessment, we tested the colocalization of CF-SAP and CF-hCT(9−32)-br with the labeled B-subunit of cholera toxin. As shown in Figure 4A, the predominant fraction of endosomal vesicles proved to be positive for both CF-SAP and cholera toxin. Further support for the concurrent localization of peptide and marker is also documented in the respective *yz* and the *xz* sections. For CF-hCT(9−32)-br colocalization with cholera toxin is demonstrated in Figure 4C. A large fraction of the vesicles proved to be positive for both the peptide and the marker. Consequently, the data point toward an involvement of lipid raft-mediated endocytosis.

Lipid rafts are defined by their cholesterol enrichment. Methyl-β-cyclodextrin (M-β-CD) extracts cholesterol from cell membranes and, therefore, disrupts lipid rafts whereas it does not disturb clathrin-dependent endocytosis (*22*, *23*, *26*, *27*). Thus, to further corroborate our hypothesis of a lipid raft-dependent internalization of extracellular CF-SAP and CF-hCT(9−32)-br, we pretreated HeLa cells with M-β-CD prior to incubation with the peptides. For control CF-hCT-(9−32) was also looked at. As shown in Figure 5A, the intracellular fluorescence was significantly reduced when HeLa cells were pretreated with M-β-CD. Taken together, our results indicate in a consistent fashion that CF-SAP and CF-hCT(9−32)-br are internalized via clathrin-independent endocytosis involving lipid rafts rather than via a clathrin-mediated pathway. Surprisingly, pretreatment with M-β-CD also reduced the internalization of the weakly cationic control peptide CF-hCT(9−32). To further confirm this outcome, we performed colocalization studies of CF-hCT(9−32) with both cholera toxin and transferrin (Figure 5B,C). Clearly, colocalization was predominant with cholera toxin but negligible with transferrin. Prior to colocalization studies with fixed cells, preliminary experiments were performed in unfixed, living cells. The two experimental protocols led to identical vesicular distributions of the two CPP as well as to identical colocalization patterns with cholera toxin (data not shown). Therefore, we followed a mild fixation protocol with 1% PFA in order to exactly terminate the experiment after 30 min and for better handling.

Involvement of Early Endosomes in the Intracellular Trafficking Pathways of CF-SAP and CF-hCT(9−32)-br. After endocytic internalization, vesicles move from the surface of the cell to intracellular compartments within the cytosol where they often undergo complex trafficking and sorting events (*35*). To determine whether our peptides were sorted into endosomes before they move to other vesicles, we examined the colocalization of both peptides with the early endosomal antigen 1 (EEA1), a protein associated with early endosomes (*39*). The overlay micrographs in Figure 6 clearly demonstrate the partial colocalization of CF-SAP (Figure 6A) or CF-hCT(9−32)-br (Figure 6B), respectively, with EEA1. Therefore, endosomal compartments are concluded to be involved in the uptake and intracellular trafficking of these cationic peptides.

We further carried out a pulse−chase experiment. The pulse referred to a brief peptide incubation period which was then followed by the chase interval where the cells were

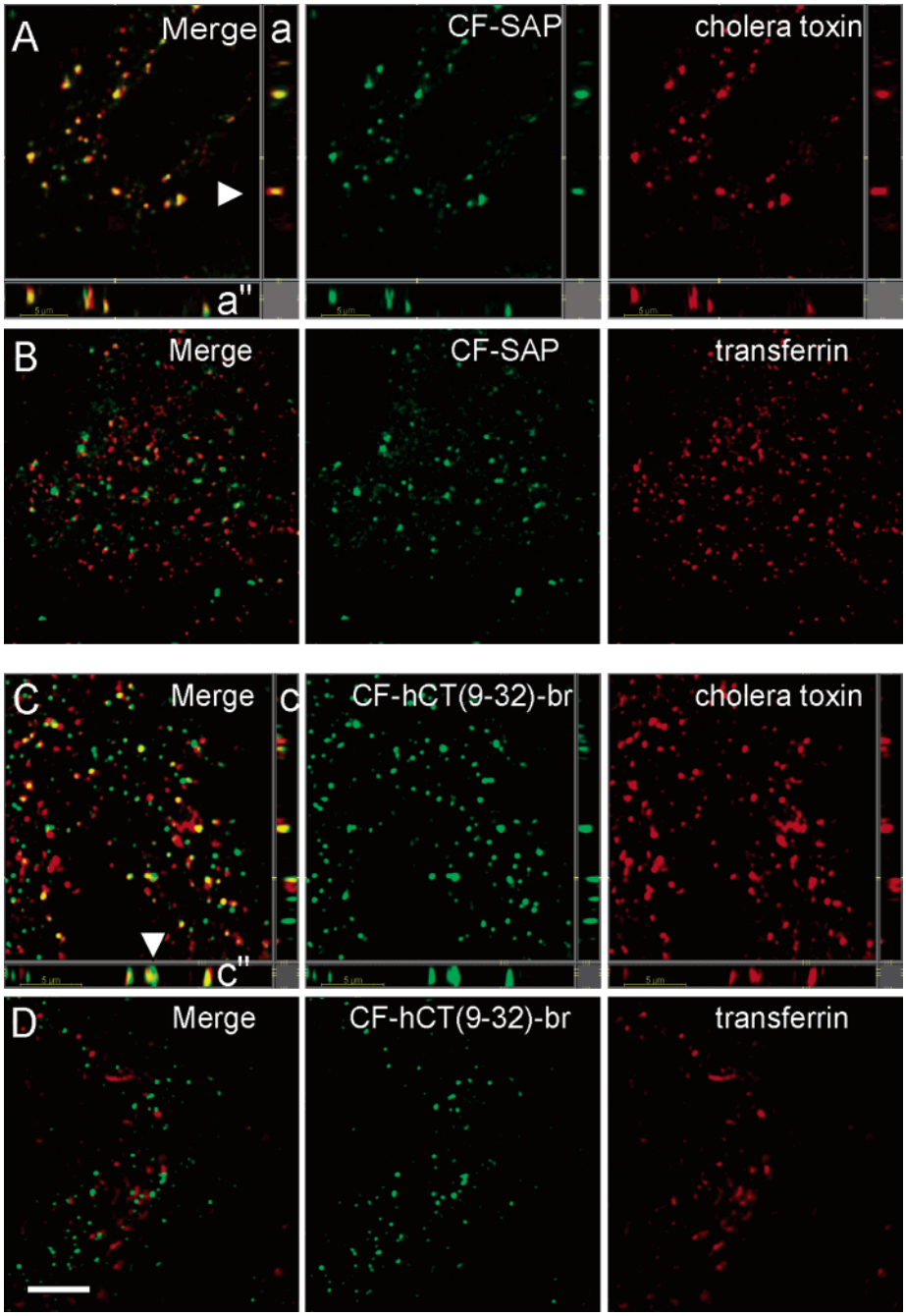

FIGURE 4: Colocalization study of CF-SAP and CF-hCT(9−32)-br with cholera toxin or transferrin, respectively. Colocalized vesicles are in yellow, peptides [CF-SAP or CF-hCT(9−32)-br] in green, and endocytosis markers (cholera toxin and transferrin) in red. Cells were incubated with 50 μM CF-SAP or 30 μM CF-hCT(9−32)-br and simultaneously stained with 10 μg/mL cholera toxin or 50 μM transferrin, respectively, for 30 min. Colocalization of both CF-SAP and CF-hCT(9−32)-br with cholera toxin suggests lipid raft-mediated endocytosis (A, C). Negative colocalization with transferrin excludes clathrin-mediated endocytosis (B, D) for both peptides. Panel A: Marked colocalization of CF-SAP with cholera toxin after 30 min. Panel B: No colocalization of CF-SAP with transferrin. Panel C: Good colocalization of CF-hCT(9−32)-br with cholera toxin. Panel D: No colocalization of CF-hCT(9−32)-br and transferrin. Illustrations a and c are *yz* projections whereas a″ and c″ represent the respective *xz* projections. The scale bar is 5 μm.

exposed to peptide-free medium. Such experiments have been suggested to allow identification of cellular trafficking pathways (*35*). After the cells were incubated with the appropriate peptide solutions for 30 min, they were washed with PBS and transferred into medium. After a 2.5 h chase period, we checked for the peptides and performed immunostaining for the presence of EEA1. The overlay (Figure 6C,D) demonstrated that only a few vesicles were still positive for CF-SAP or CF-hCT(9−32)-br, respectively, and EEA1. This leads to the conclusion that, after a 3 h incubation time, major fractions of the two investigated cationic peptides no longer remained in the early endosome compartments but may have moved to other compartments within the cytosol.

Subsequently, we tested the hypothesis that the peptides may have entered the endoplasmic reticulum or the Golgi network using a colocalization technique. None of the related experiments yielded unequivocal results, neither in favor nor against this hypothesis (data not shown).

Cell Proliferation and Viability. As measured by XTT assays, proliferation and cellular viability of HeLa cells were not affected by incubation with hCT(9−32) or hCT(9−32)-br. We tested concentrations up to three times higher than

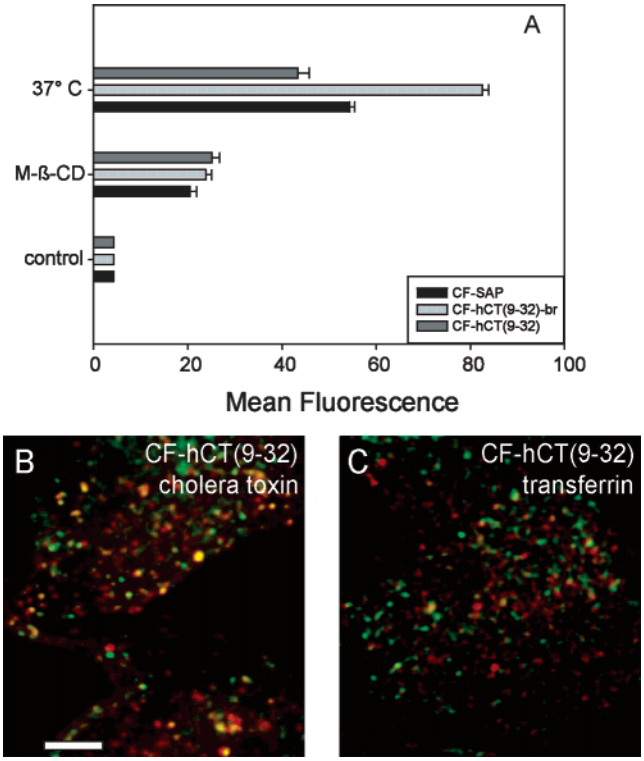

FIGURE 5: Panel A: Inhibition of lipid raft-mediated endocytosis of CF-SAP, CF-hCT(9−32)-br, and CF-hCT(9−32) in HeLa cells as determined by FACS analysis. HeLa cells were incubated for 3 h at 37 °C with 50 μM CF-SAP (black bars), CF-hCT(9−32)-br (light gray bars), or CF-hCT(9−32) (dark gray bars) (both 30 μM). Inhibition of lipid raft-mediated endocytosis was performed by pretreating cells with 10 mM methyl-β-cyclodextrin (M-β-CD) before incubation with the peptide solutions. Nontreated cells were used as control cells. Panels B and C: Colocalization of CF-hCT(9−32) with cholera toxin (B) or transferrin (C), respectively. Colocalized vesicles are in yellow, CF-hCT(9−32) is in green, and endocytosis markers (cholera toxin and transferrin) are in red. Cells were incubated with 30 μM CF-hCT(9−32) and simultaneously stained with 10 μg/mL cholera toxin or 50 μM transferrin, repectively, for 30 min. Panel B: Marked colocalization of CF-hCT(9−32) with cholera toxin after 30 min. Panel C: No colocalization of CF-hCT(9−32) with transferrin. The scale bar is 5 μm.

the concentrations used in this study. No relevant reduction of cell viability could be detected (data not shown).

DISCUSSION

The cell membrane poses a substantial hurdle to the use of pharmacologically active biomacromolecules that are not per se actively translocated into cells. To overcome this limitation, a variety of CPP has been developed (*2*−*4*) and evaluated for their ability to deliver therapeutics into cells that otherwise cannot translocate cellular membranes. Because of their highly cationic nature, concerns have been raised as to their cytotoxicity in cell culture experiments (*31*, *40*), in light of a theoretical extrapolation to a clinical scenario.

In recent years, the translocation mechanisms of major CPP have been controversially discussed and are not yet fully understood. An early assumption that oligocationic CPP such as Tat peptides and penetratin translocate via a direct, energy-independent mechanism (*2*, *3*, *15*) has been widely questioned and concluded to result from fixation artifacts and other experimental shortcomings. The recent reevaluation of

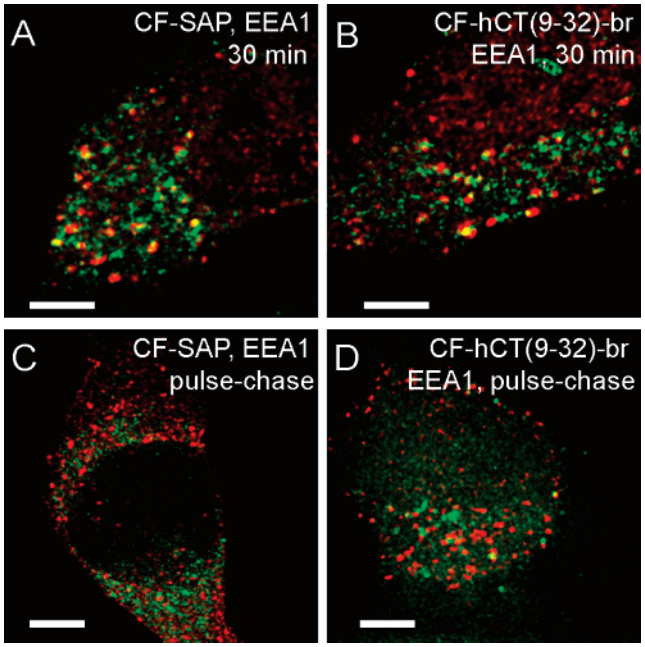

FIGURE 6: Colocalization of CF-SAP and CF-hCT(9−32)-br with EEA1. Colocalized vesicles are visible in yellow, whereas peptides [CF-SAP or CF-hCT(9−32)-br] are visible in green and the endocytosis marker (EEA1) is in red. Panel A shows colocalization of CF-SAP (50 μM) with the endosomal endocytosis marker EEA1 (10 μg/mL). Panel B: Merge of 30 μM CF-hCT(9−32)-br and EEA1 (10 μg/mL). The incubation time with peptides was 30 min. Panels C and D represent pulse−chase studies of EEA1 and CF-SAP (C) or CF-hCT(9−32)-br (D) with an incubation time with peptides of 30 min followed by a chase period of 2.5 h. The scale bar is 5 μm.

the involved mechanisms is about to converge into the opposite: an energy-dependent, endocytic uptake mechanism, as demonstrated for Tat and penetratin (*16*, *17*). More recent studies dissected the endocytic uptake of the Tat peptide in greater detail (*41*, *42*).

To preclude any artifacts through cell fixation in our study, we abstained from fixation of CLSM and FACS samples and performed our experiments in living cells without fixation, except for the colocalization studies where a mild fixation protocol was performed to stop the experiment after an exact time point of 30 min and for better handling conditions. With this protocol, we saw no differences between fixed and unfixed samples as previously also observed by Pichon et al. (*43*). Clear distinction between intracellular and membrane-bound fluorescence in our FACS experiments was achieved in a twofold way: we (i) digested eventually adsorbed CPP by trypsinization with trypsin−EDTA (*16*) and (ii) quenched remaining extracellular fluorescence by Trypan blue (*44*, *45*).

We analyzed the uptake mechanism of two novel oligocationic CPP, CF-SAP (*30*−*32*) and CF-hCT(9−32)-br (*29*). Compared with other CPP, CF-SAP and CF-hCT(9−32)-br may offer several advantages including their good solubility in water, the documented lack of cytotoxicity, and, in case of CF-SAP, its nonviral origin (*29*, *31*). In previous studies, truncated linear sequences of human calcitonin could translocate plasma membranes and demonstrated punctuated vesicular-type, cytoplasmic distribution after incubation (*11*). The translocation efficiency of its branched derivative, CF-hCT(9−32)-br, turned out to be superior to the corresponding

linear peptide CF-hCT(9−32), used as control peptide in this study. Most probably, the enhancement resulted from the oligocationic NLS sequence in the side chain of CF-hCT-(9−32)-br, representing a well-known import sequence (*46*).

For both peptides, CF-SAP and CF-hCT(9−32)-br, we observed marked cellular uptake in HeLa cells with the vesicular distribution pattern in the cytoplasm typical for an endocytic translocation mechanism (*47*). Consistent with this finding, translocation occurred through a temperature- and energy-dependent pathway. Uptake occurred at 37 °C but was strongly reduced at 4 °C in coincidence with an accumulation of peptide on the cell surfaces forming small patches, presumably membrane-associated aggregates. Consistent with this observation is that ATP depletion impaired the translocation of both CF-SAP and CF-hCT(9−32)-br in HeLa cells.

Addition of heparin reduced the translocation rate in a way that correlated with their content of cationic amino acids, in that inhibition was much more efficient with CF-hCT(9−32)-br than with the less cationic CF-SAP. This supports the assumption that the primary interaction of the two peptides with the cell surface occurred via sulfated proteoglycans of the negatively charged ECM through electrostatic binding. Our observation is in agreement with findings for other oligocationic CPP (*14*). Consistently, the weakly cationic control peptide CF-hCT(9−32) was unaffected by heparin, indicating absence of significant interaction with the ECM.

There are several endocytic pathways for CPP possible through which trafficking into cytoplasmic compartments may proceed, including endocytosis via clathrin-coated pits (*19*, *20*) and endocytosis via lipid rafts (*24*, *25*). In the present study, we could reveal that the two investigated oligocationic CPP, CF-SAP and CF-hCT(9−32)-br, were internalized by clathrin-independent endocytosis via lipid rafts. The rationale of our experiments was based on the following considerations: (i) transferrin is a marker for clathrin-mediated endocytosis and is excluded from lipid rafts (*24*, *25*), (ii) the function and organization of lipid rafts are perturbed by depletion of cholesterol from membranes, providing a tool to discriminate between raft- and non-raft-dependent processes (*22*, *23*, *27*), and (iii) cholera toxin subunit B follows a clathrin-independent pathway from the plasma membrane to the Golgi (*22*, *23*, *26*). Colocalization studies with transferrin gave no indication for an internalization pathway of CF-SAP or CF-hCT(9−32)-br via clathrin-coated pits. Instead, we detected marked colocalization of both CF-SAP and CF-hCT(9−32)-br with cholera toxin. This confirms the existence of a clathrin-independent internalization pathway of the two peptides. Further, depletion of cholesterol by methyl-β-cyclodextrin (M-β-CD) (*48*) drastically reduced the amount of intracellular fluorescence, suggesting an involvement of lipid rafts. Taken together, our findings led us to the proposition of both CF-SAP and CF-hCT(9−32)-br being internalized via a clathrin-independent mechanism that originates from plasma membrane lipid rafts. Our findings corroborate with recent observations of other groups on already established cationic peptides revealing comparable clathrin-independent mechanisms to originate from plasma membrane lipid rafts as the underlying translocation mechanisms, namely, for Tat fusion proteins (*49*) and the equally oligocationic antimicrobial peptide LL-37 (*50*). Nevertheless, as shown for the unbranched CF-hCT(9−32) control peptide, the lipid raft-mediated pathway is not exclusive for oligocationic peptides. As demonstrated by the outcome of the M-β-CD inhibition study in combination with cholera toxin colocalization, lipid rafts represent the preferred pathway of uptake even for the weakly cationic CF-hCT(9−32). Remarkably, even with one positive charge only and not subject to significant interaction with the ECM, the cellular access of CF-hCT(9−32) occurred via the lipid raft-mediated pathway. This is in disagreement with recent proposals of a mandatory combination of proteoglycan interaction and lipid raft-dependent uptake, as proposed for Tat fusion proteins (*49*) and the human antimicrobial peptide LL-37 (*50*). Our results suggest that irrespective of charge and interaction with the ECM, the lipid raft-mediated pathway represents the preferred port of entry for various classes of CPP and is not exclusive for oligocationic peptides.

On its way into the cell cholera toxin starts by binding the ganglioside GM1 that associates with lipid rafts on the cell surface (*24*, *51*). Following endocytic internalization, cholera toxin exploits the retrograde pathway and can be found in early and recycling endosomes, in the Golgi apparatus, and in the endoplasmic reticulum (*52*−*54*). To get insight into the intracellular trafficking pathways of CF-SAP and CF-hCT(9−32)-br and to determine whether these peptides are sorted in endosomes before they move to other vesicles, we performed colocalization studies with the early endosomal antigen 1 (EEA1), a protein associated with early endosomes. The overlay revealed that early endosomes are involved in the uptake and initial intracellular trafficking of the two oligocationic peptides. Additionally, from the result of a pulse−chase study, we conclude that, after an incubation time of 3 h, most of the internalized CF-SAP or CF-hCT-(9−32)-br was no longer present in early endosomes but moved to other compartments within the cytosol or was metabolically degraded. Studies on the metabolic stability of the two peptides revealed pronounced stability of CF-SAP excluding metabolic degradation as an explanation of poor endosomal retention (C. Foerg, unpublished data). Equipped with an endosomal cleavage site in the side chain, CF-hCT(9−32)-br revealed a significantly lower metabolic stability (C. Foerg, unpublished data). Therefore, as an alternative, high susceptibility to intracellular proteases in combination with endosomal escape of the generated fragments may explain the loss of endosomal fluorescence observed with CF-hCT(9−32)-br.

Fischer et al. hypothesized about the involvement of a retrograde pathway in cellular trafficking of cationic CPP (*41*) via the Golgi apparatus to the cytosol. In the current study, however, we could not visualize CF-SAP or CF-hCT-(9−32)-br beyond endosomal vesicles, such as in the Golgi or in the endoplasmic reticulum. This could possibly indicate that translocation occurs with a minor fraction of the internalized molecules only, as was also the case for ricin (*55*). For the further development of efficient CPP it will be of crucial importance to dissect the intracellular trafficking pathways further and to determine the final cellular target compartment(s) of each CPP. Preliminary data from our group (C. Foerg, unpublished data) suggest that cellular translocation is highly cell-type specific and that the stage of proliferation has an important impact on CPP translocation rates and mechanisms. In parallel to the tightening of the

paracellular barrier formed by the tight-junctional complex, confluency and cellular differentiation appear to evoke tighter plasma membrane barriers against CPP and result in lower translocation rates. This phenomenon is subject to further studies in our laboratories at the moment.

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