A Stepwise Dissection of the Intracellular Fate of Cationic Cell-penetrating Peptides*

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The role of endosomal acidification and retrograde transport for the uptake of the highly basic cell-penetrating peptides penetratin, Tat, and oligoarginine was investigated. The effect of a panel of drugs that interfere with discrete steps of endocytosis or Golgi-mediated transport on uptake and cellular distribution of fluorescein-labeled peptide analogues was probed by confocal microscopy, flow cytometry, and fluorescence spectroscopy of whole cell lysates. The analogues were carried out in MC57 fibrosarcoma cells and in HeLa cells. While MC57 fibrosarcoma cells showed some vesicular fluorescence and a pronounced cytoplasmic fluorescence, in HeLa cells little cytoplasmic fluorescence was observed. In MC57 cells the inhibitors of endosomal acidification chloroquine and bafilomycin A1 abolished the release of the peptides into the cytoplasm. Release into the cytosol preserved endosomal integrity. In addition, cellular uptake of the peptides was inhibited by brefeldin A, a compound interfering with trafficking in the trans-Golgi network. In contrast, nordihydroguaiaretic acid, a drug that stimulates the rapid retrograde movement of both Golgi stacks and trans-Golgi network to the endoplasmic reticulum, promoted a cytoplasmic localization of Tat peptides in peptide-pulsed HeLa cells. The effects of these drugs on trafficking shared characteristics with those reported for the trafficking of plant and bacterial toxins, such as cholera toxin, which reach the cytoplasm by means of retrograde transport. A sequence comparison revealed a common stretch of 8–10 amino acids with high sequence homology to the Tat peptide. The structural and functional data therefore strongly suggest a common mechanism of import for cationic cell-penetrating peptides and the toxins.

The introduction of membrane-impermeable molecules into mammalian cells has become a key strategy for the investigation of intracellular processes (1). Peptide-mediated import has been attracting growing attention as a delivery technology during the last decade (for reviews, see Refs. 2 and 3). Linkage of so-called cell-penetrating peptides (CPPs)1 to other molecules mediates the non-invasive import of these cargo molecules into cells ex vivo as well as in whole animals (4, 5). Cargo molecules have been peptides (6, 7), proteins as large as 120 kDa (5, 8), oligonucleotides (9), plasmids (10), peptide nucleic acids (11), and even nanoparticles (12).

Peptide cargos delivered by conjugation to CPPs include pseudosubstrates (13), competitive inhibitors of an enzyme active site (14), compartment-specific localization sequences (15), structural mimetics of interaction domains (16), and epitopes for presentation by major histocompatibility complex class I molecules (4, 17). The advantages of peptide-based functional analyses in cell biology are the accessibility of large collections of different compounds by well established automated procedures (18) and a rational approach to the generation of biologically active compounds based on available structural information of interaction domains. Structure-function relationships of intracellular interaction domains have been analyzed by testing a series of different peptides fused to cell-penetrating peptides (19). The delivery of exogenous antigens into the major histocompatibility complex class I processing pathway using CPPs has been presented in vitro and in whole animals (4, 17). On dendritic cells CPP-epitope constructs have been shown to enable prolonged antigen presentation (20). CPPs therefore have the promise to represent a widely applicable means to enhance immune responses against cancer and infectious diseases.

A total of about 20 different peptide delivery vectors have been described so far, the majority of which were identified as structural determinants mediating cellular internalization of proteins (3). While some of these peptides are purely cationic, others are amphipathic with a large fraction of basic residues, and again others are fully hydrophobic. These peptides vary in length from about 9 to more than 30 amino acids (3).

Three highly basic import peptides, the Drosophila Antennapedia homeodomain-derived penetratin peptide (21), the HIV-1 Tat-derived peptide (22), and the oligoarginine peptides (23) have been used widely in many of the applications described above. The penetratin peptide and the HIV-1 Tat peptide have been identified as protein transduction domains of their respective proteins, while the oligoarginine peptides (among them the nona-arginine peptide R9) have been developed based on structure-activity relationships of the HIV-1 Tat peptide.

Despite their broad acceptance as molecular carriers, the mechanism of internalization of CPPs and CPP-cargo constructs is not well understood. The uptake of the penetratin and the HIV-1 Tat peptide had originally been described to be insensitive to low temperature (21, 22, 24) and to inhibitors of salt solution; HIV-1, human immunodeficiency virus, type 1; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; NDGA, nordihydroguaiaretic acid; PBS, phosphate-buffered saline; RT, room temperature; TGN, trans-Golgi network; TOF, time-of-flight.

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1 The abbreviations used are: CPP, cell-penetrating peptide; Antp, Antennapedia; DMF, N,N-dimethylformamide; Fluoro, 5(6)-carboxyfluorescein; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HBSS, Hank’s buffered
endocytosis (22, 25). Penetratin was also demonstrated to transverse a pure lipid bilayer (26) without forming pores (26, 27). In summary, these results were consistent with the theory of a direct translocation of the cationic peptides through the plasma membrane (21, 22, 28).

Recent data demonstrated, however, that earlier interpretations of cell biological experiments may have suffered from artificial uptake of CPPs caused by fixation of cells. Endocytosis is clearly involved in the internalization of the HIV-1 Tat peptide (29). For Tat fusion proteins it was demonstrated that cellular internalization occurs through a temperature-dependent endocytic pathway that originates from lipid rafts and follows caveolar endocytosis (30). Inhibitors of metabolism or endocytosis, such as cytochalasin D, were demonstrated to impair uptake of penetratin (31). Recently the penetratin and the Tat peptide were shown to promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans (32).

With a picture of an endocytic uptake mechanism emerging, the implications of the endosomal pathway for functional cell biological studies using CPPs need to be readdressed. The biological activity exhibited by CPPs in cell biological applications (6) is fully consistent with intact CPP-constructs reaching the cytosol. However, the accumulation of CPPs in the endocytic compartment (29–31) raises the question to which degree and by which mechanism internalized CPPs reach the cytosol. A rational design of more effective CPPs can only be achieved if the mechanism of uptake is fully understood.

In this study we addressed these questions by investigating the effect of drugs that interfere with distinct steps of the endosomal pathway and Golgi trafficking. To study a potential cell type dependence of the peptide trafficking, adherently growing MC57 fibrosarcoma cells and HeLa cells were examined. Both cell lines showed marked differences in the intra-cellular distribution of fluorescein-labeled CPPs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Standard chemicals for peptide chemistry were obtained from Novabiochem, Senn Chemicals (Dielsdorf, Switzerland), and Orpegen Pharma (Heidelberg, Germany). The isomeric mixture of 5/6-carboxyfluorescein was from Fluka, and AlexaFluor 647-dextran (anionic, molecular mass, 10,000 Da) and Bodipy TR ceramide were obtained from Fluka (Deisenhofen, Germany) and Merck; solvents were pure grade.

**Automated peptide synthesis** was performed by a Merck Biosystems (Molsheim, France) fitted with a Plan-Apochromat 63x/1.4 objective. Peptide amides were Fmoc-protected amino acids (12-fold excess) were coupled by in situ activation using N,N′-diisopropyl carbodiimide/1-hydroxybenzotriazol (50:1), Bodipy TR Ceramide, and AlexaFluor 647-dextran were obtained from Molecular Probes (Eugene, Oregon), and AlexaFluor 647-dextran, fluorescence of 5000 vital cells was acquired. Vital cells were gated based on forward scatter. For pulse/chase experiments, MC57 cells were seeded at a density of 200,000/well in 24-well plates (Sarstedt, Nuembrecht, Germany). For double detection of fluorescein-labeled peptides and AlexaFluor, flow cytometry was performed on an Inreads 300010 flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey) fitted with a Plan-Apochromat 63x/1.4 objective. Peptide amides were synthesized on Rink amide resin (Rapp Polymere, Tubingen, Germany).

Peptides were cleaved off the resin by treatment with trifluoroacetic acid/trisopropylsilane/ethanedithiol/H₂O (9:2:5:2:5:2:5:2:5, v/v/v/v/v/v/v/v) for 4 h. Crude peptides were precipitated by adding cold diethyl ether (−20 °C). The precipitated peptides were collected by centrifugation and resuspended in 500 μl of cold diethyl ether. This procedure was repeated twice. Finally peptides were dissolved in tert-butyl alcohol/H₂O (4:1, v/v) and lyophilized.

**Labeling of Peptides with Carboxyfluorescein**—Amino-terminal labeling of peptides with 5(6)-carboxyfluorescein was essentially performed as described previously (33). Fmoc-protected, resin-bound peptides were reacted with 5 eq of (5(6))-carboxyfluorescein. N,N′-diisopropyl carbodiimide, and 1-hydroxynbenzotriazol, each in DMF for 16 h in 2-ml syringes on a shaker at RT. Reactions were stopped by washing the resin three times each with DMF, methanol, dichloromethane, and diethyl ether. Subsequently peptides were treated with piperdine/DMF (1:4, v/v) to remove ester-bound carboxyfluorescein (33). Completeness of amine acylation was confirmed using the Kaiser test (34).

**MALDI-TOF MS of Synthetic Peptides**—1 μl of 2.5-dihydroxyacetophenone matrix (20 mg of 2.5-dihydroxyacetophenone, 5 mg of ammonium citrate in 1 ml of isopropyl alcohol/H₂O (4:1, v/v)) was mixed with 1 μl of each sample (dissolved in acetonitrile/water (1:1) at a concentration of 1 mg/ml) on a gold target. Measurements were performed using MALDI time-of-flight system (G2025A, Hewlett-Packard, Waldbronn, Germany). For signal generation 20–50 laser shots were added up in the single shot mode.

**Peptide Stock Solutions**—Carboxyfluorescein-labeled peptides were dissolved in Me2SO at concentrations of 10 mM. These stock solutions were further diluted 1:20 in double distilled H₂O. Peptide concentrations were determined by UV/visible spectroscopy of a further 1:100 dilution in 0.1 M Tris/Cl buffer (pH 8.8) with absorptions measured at 492 nm and assuming a molar extinction coefficient of 75,000 liters/(mol·nm).

**Cell Culture**—The adherent MC57 fibrosarcoma cell line (35) and HeLa cells were grown in a 5% CO₂ humidified atmosphere at 37 °C in RPMI 1640 medium with 15% foetal calf serum (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (PAN Biotech), 100 units/ml penicillin, and 100 μg/ml streptomycin (Biochrom, Berlin, Germany). Both cell lines were passaged by trypsinization with trypsin/EDTA (0.05%/0.02%, w/v) (Biochrom) in PBS every 3rd–4th day.

**Flow Cytometry**—MC57 or HeLa cells were seeded at a density of 50,000/well in 24-well plates (Sarstedt, Nümbrecht, Germany) in serum-containing RPMI 1640 medium. One day later, the cells were washed with serum-free RPMI 1640 medium and incubated in 200 μl of serum-free RPMI 1640 medium containing the appropriate inhibitors. After the indicated period of time, peptides were added as described using a 1:100 dilution of the indicated concentration. Each condition was tested in duplicate. After a 2-h incubation, cells were washed with PBS, detached by trypsinization for 10 min, suspended in ice-cold PBS containing 0.1% (w/v) bovine serum albumin, and measured immediately by flow cytometry. The fluorescence of 5000 vital cells was acquired. Vital cells were gated based on sideward scatter and forward scatter.

For pulse/chase experiments, MC57 cells were washed three times with PBS after 2 h of peptide incubation and then incubated with 500 μl of serum-free RPMI 1640 medium for an additional 3 h. Cells were then washed with PBS, detached by trypsinization for 10 min, suspended in ice-cold PBS containing 0.1% (w/v) bovine serum albumin, and measured by flow cytometry as described above. To compare the results of the pulse/chase values, fluorescence intensities of fluorescein calibration beads (Mobitech) were also acquired for each series of measurements.

**Confocal Laser Scanning Microscopy**—Confocal laser scanning microscopy was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Göttingen, Germany) fitted with a Plan-Apochromat objective (×40, numerical aperture 1.3). All measurements were performed with living, non-fixed cells.

MC57 cells were seeded at a density of 10,000/well in 8-well chambered cover glasses (Nunc, Wiesbaden, Germany). Two days later, before addition of inhibitors or peptides, cells were washed once with serum-free RPMI 1640 medium. Bafilomycin A1 was added at a concentration of 500 nM in 200 μl of serum-free RPMI 1640 medium 30 min before addition of peptides. After 2 h of incubation with peptides, images were acquired immediately at RT with excess peptide in the medium.

For double detection of fluorescein-labeled peptides and AlexaFluor.
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647-dextran, the 488 nm line of an argon ion laser and the light of a 633 nm helium/neon laser were directed over an HFT UVV488/633 beam splitter, and fluorescence was detected using an NFT 545 beam splitter in combination with a BP 505–530 band pass filter for fluorescence detection and an LP 650 long pass filter for AlexaFluor 647 detection. Peptides and AlexaFluor 647-dextran were added as indicated under “Results.” After 2 h cells were washed three times with serum-free RPMI 1640 medium followed immediately by confocal microscopy at RT. For the induction of retrograde transport, after 2 h of incubation with peptide and dextran, cells were washed three times with serum-free RPMI 1640 medium following by incubation with 25 μM NDGA for 3 h in RPMI 1640 medium.

For double detection of fluorescein-labeled peptides and Bodipy TR ceramide, the 488 nm line of an argon ion laser and the light of a 543 nm helium/neon laser were directed over an HFT UVV488/543 beam splitter, and fluorescence was detected using an NFT 545 beam splitter in combination with a BP 505–530 band pass filter for fluorescein detection and an LP 560 long pass filter for Bodipy detection.

Staining of the Golgi complex in living cells with fluorescent ceramide was essentially performed as described by the supplier. Briefly, MC57 and HeLa cells were seeded at a density of 10,000/well in 8-well chambered cover glasses. Two days later, cells were washed once with ice-cold Hank’s buffered salt solution + 10 mM HEPES (pH 7.4) (HBSS/HEPES) and then incubated with HBSS/HEPES (containing 5 μM sphingolipid and 5 μM bovine serum albumin prepared as described by the supplier) for 30 min on ice and in the dark. Cells were rinsed three times with ice-cold HBSS/HEPES and incubated with the indicated peptide in serum-free medium for 1 h at 37 °C in the incubator. Confocal images were then acquired immediately at RT with excess peptide in the medium.

MALDI-TOF MS of Peptide-containing Cell Culture Supernatant—A confluent layer of MC57 cells in a 25-cm² tissue culture flask was washed once with serum-free RPMI 1640 medium and incubated with 1 ml of serum-free RPMI 1640 medium containing 30 μM Fluo-Tat. After 2 h the cells were detached using 5 mM EDTA/PBS, transferred into a fresh tube, and washed twice with 10 ml of PBS. EDTA/PBS was used instead of trypsin to exclude that peptide fragments were generated by residual amounts of trypsin during the chase period. Peptides were then suspended in 500 μl of serum-free RPMI 1640 medium and incubated with 1 ml of the sample on a gold target. For signal generation the samples were acquired in the presence of peptide in the medium.

Results.

Table 1: Primary structures of the cell-penetrating peptides used in this study

| Peptides     | Sequences                |
|--------------|--------------------------|
| Fluo-Antp    | Fluo-RQIKWFQRRMKWKK-CONH₂ |
| Fluo-R9      | Fluo-RRRRRRRR-CONH₂       |
| Fluo-Tat     | Fluo-YGRKKRRQRRR-CONH₂    |

Then Fluo-Antp was added, and after 2 h of incubation, cells were washed twice with PBS, detached by trypsinization for 15 min, transferred into a fresh tube, and washed twice with 10 ml of PBS (to remove trypsin and trypsinized peptide). Cells were then lysed in 200 μl of Nonidet P-40 lysis buffer (0.5% (w/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.0, containing protease inhibitor mixture (Roche Diagnostics)). The lysates were then sonicated and centrifuged for 30 min at 4 °C and 14,000 rpm. Fluorescein concentrations were determined in the supernatant using an L550B spectrofluorometer (PerkinElmer Life Sciences) with excitation at 492 nm and detection of emission at 520 nm. The protein content of the lysates was determined using a commercially available Bradford protein assay kit (Bio-Rad). Each condition was tested in duplicate.

RESULTS

Cellular Uptake of Penetratin, R9, and Tat Peptides—Considering two recent studies demonstrating the involvement of endocytosis in the uptake of the R9 peptide, the HIV-1 Tat peptide (29), and Tat fusion proteins (30), we intended to dissect in detail the cellular mechanisms involved in the uptake of cationic CPPs (Table I) and their release into the cytosol. Initially the effect of wortmannin, bafilomycin A1, and chloroquine as inhibitors of endocytosis and endosomal acidification on the uptake of fluorescently labeled CPPs was assessed by flow cytometry of living cells. While wortmannin affects early endosome fusion by inhibition of the phosphatidylinositol 3-kinase (37), chloroquine (38, 39) and bafilomycin A1 (40, 41) inhibit endosomal acidification. While chloroquine diffuses across membranes and binds protons, bafilomycin A1 is a highly potent and selective inhibitor of vacuolar H⁺-ATPases (40). In addition to R9 and the Tat peptide, the penetratin peptide was included in this comparison.

The distortion of uptake experiments by peptides that were only associated with the plasma membrane but had not been internalized by the cells was avoided by treating the cells with trypsin prior to flow cytometry (29). While Fluo-R9 and Fluo-Antp were taken up efficiently to comparable levels, the intracellular fluorescence of Fluo-Tat was less than 10% of that of cells treated with the other two peptides (Fig. 1). For Fluo-R9 and Fluo-Antp, all three inhibitors led to a reduction of the cellular fluorescence by about 50%. In contrast, for the Fluo-Tat peptide only a small effect of wortmannin was observed, while for chloroquine and bafilomycin A1 intracellular fluorescence increased.

Impact of Bafilomycin A1 on the Cellular Distribution of Fluorescently Labeled CPPs—To explain the contrasting results for Fluo-Tat and the other two peptides obtained by flow cytometry, the effect of bafilomycin A1 on the intracellular localization of the peptides was addressed using confocal laser scanning microscopy. Live cell confocal microscopy was performed after 2 h of incubation with peptides at 37 °C. Images were acquired in the presence of peptide in the medium to avoid loss of cellular fluorescence due to wash out (42). To compensate for the lower cellular fluorescence of Fluo-Tat, this peptide was applied at a 5-fold higher concentration (5 μM). For all three CPPs, both a distinct vesicular staining and cytoplasmic localization were detected (Fig. 2, A, B, and C, upper panels). Cytoplasmic fluorescence was weakest for the Fluo-Tat peptide. For the Fluo-R9 peptide a slight nuclear enrichment of...
fluorescein fluorescence in MC57 cells could be observed (Fig. 2C).

When bafilomycin A1 was added to the incubation medium, no cytoplasmic fluorescence was present for any of the three CPPs. Instead the vesicular staining was enhanced for all three peptides (Fig. 2, A–C, lower panels). Using chloroquine as an inhibitor of endosome maturation, essentially the same effect was observed. However, this inhibitor led to the formation of large vesicular structures (data not shown).

**Exit of Fluo-Tat Fragments from the Cytosol**—While bafilomycin A1 and chloroquine led to a reduced uptake of Fluo-Antp and Fluo-R9, for Fluo-Tat cellular fluorescence increased. This result is in apparent disagreement with a common internalization mechanism of Tat and R9 (25). Bafilomycin A1 leads to the accumulation of both peptides in vesicular structures thereby inhibiting their release into the cytoplasm. For this reason the increase in cellular Fluo-Tat fluorescence caused by bafilomycin A1 as observed by flow cytometry (Fig. 1B) might be due to the ability of Fluo-Tat or fluorescent proteolytic fragments to leave the cells rapidly after entry into the cytosol in the absence of bafilomycin A1. To test this, MC57 cells were pulsed with the three fluorescently labeled CPPs for 2 h, washed, and then incubated with peptide-free medium for an additional 3 h (42). The Fluo-Tat peptide was again applied at a 5-fold higher concentration (5 μM). Cellular fluorescence of MC57 cells after pulse and pulse/chase was determined by flow cytometry (Fig. 3A). Both the Fluo-Antp and the Fluo-R9 peptides were not chased out of the cells efficiently. However, for Fluo-Tat after a 3-h chase period, only very little fluorescein fluorescence remained associated with the MC57 cells. Two different explanations were considered as the basis for this experimental outcome. First, in contrast to penetratin and oligoarginine, the Tat peptide itself could possess the ability to exit the cells by crossing the plasma membrane. Second, instead of the intact full-length peptide, fluorescent fragments generated by proteolytic breakdown could leave the cell by diffusion through the plasma membrane. To answer this question, supernatants of MC57 cells after the chase period were desalted, concentrated, and analyzed by mass spectrometry (Fig. 3B). Peptide fragments consisting of an amino-terminal carboxyfluorescein and 1–6 amino acids were specifically detected in chase supernatants of peptide-pulsed cells. Intact Tat peptide could not be detected. These data strongly indicate that the loss of fluorescence was due to exit of fluorescent peptide fragments during the 3-h chase period.

**Effect of CPPs on Endosomal Integrity and Intracellular Peptide Stability**—Having shown that inhibitors of endosomal acidification inhibited the release of cationic CPPs into the cytosol, we next asked whether entry into the cytoplasm involves destabilization of endosomal membranes after endosomal acidification. Such a mechanism has been described, e.g., for peptides containing the amino-terminal sequence of influenza virus hemagglutinin HA-2 (43). MC57 cells were incubated with medium containing anionic high molecular mass (10,000 Da) AlexaFluor 647-dextran in the absence or presence of Fluo-Antp for 2 h. Dextran are internalized by fluid-phase endocytosis and accumulate in vesicles that appear as bright spots (43) (Fig. 4B). When MC57 cells were co-incubated with AlexaFluor 647-dextran and Fluo-Antp the AlexaFluor 647-dextran partially co-localized with Fluo-Antp in vesicular structures. However, the morphology of the AlexaFluor-dextran spots did not change, although the Fluo-Antp peptide clearly localized to the cytoplasm (Fig. 4, F and G), indicating that Fluo-Antp does not disrupt endosomal membranes. To address cellular peptide integrity, after 2 h of peptide incubation, cell lysates were prepared and subjected to MALDI-TOF MS analysis essentially as described previously (36). Comparison of isolates from peptide-loaded and peptide-free cells revealed two peptide-specific signals, a peak at [M + H]+ = 2605.3 Da and one at [M + H]+ = 5313.3 Da. The former corresponds to the intact Fluo-Antp peptide with a calculated [M + H]+ of 2605.1 Da, and the latter most likely corresponds to Fluo-R-OH with a calculated [M + H]+ of 5335.5 Da (Fig. 4F).

**The Subcellular Distribution of CPPs Is Cell-type Dependent**—CPPs have been applied for loading of molecules into a variety of different cell lines, including tissue culture cells and primary cells growing adherently as well as in suspension. A limited number of studies have demonstrated that for a given CPP-containing molecule uptake efficiency is cell type-dependent (44, 45). In these previous studies whole cell read-outs (44) or microscopy of fixed cells (44, 45) was utilized for comparison of uptake efficiencies. Using confocal microscopy of living cells,
we determined whether a cell type dependence could be observed for the subcellular distribution of fluorescein-labeled CPPs. HeLa cells were chosen as a second cell line because in the study of Richard et al. (29) this cell line was also investigated. In contrast to MC57 cells (Fig. 5A, upper panels) in HeLa cells at a concentration of 1 μM the Flu-o-Antp peptide was only detectable in vesicular structures (Fig. 5A, center panels) as was the case for the Flu-o-R9 peptide (data not shown). The same difference in localization was observed when both cell lines were co-cultivated and co-incubated with the Flu-o-Antp peptide to rule out any artifacts from cultivation conditions (Fig. 5A, lower panels).

At this point two possibilities were considered as the basis for this observation. First, uptake into HeLa cells may be less efficient than uptake into MC57 cells. Second, intracellular trafficking may be different in both cell lines. Analysis of cellular fluorescence of Flu-o-Antp by flow cytometry yielded comparable intracellular fluorescence for both cell lines (Fig. 5B). However, vesicular accumulation of fluorescein-labeled peptides may lead to concentration quenching of fluorescein fluorescence (46). Moreover the fluorescence of fluorescein is strongly pH-dependent, compromising a quantitative comparison of the amounts of peptide localized in acidic vesicles with that localized in the cytoplasm. For this reason, whole cell lysates of MC57 and HeLa cells loaded with peptide were prepared, and the amount of fluorescein was determined by fluorescence emission spectroscopy (Fig. 5C). Despite the very similar cellular fluorescence observed by flow cytometry, analysis of lysates revealed that HeLa cells internalized 3 times more peptide than MC57 cells (Fig. 5C). For this reason, the differences in the distribution of cellular fluorescence do indeed reflect differences in the intracellular trafficking of peptide in these two cell lines. However, for treatment with bafilomycin A1, a similar reduction of cellular fluorescence was observed for both cell lines, indicating that the differences in localization reflect quantitative differences in trafficking rather than a different mechanism.

**Impact of Golgi-disrupting Agents on the Uptake and Distribution of Fluorescently Labeled CPPs—** Having shown that release of cationic CPPs into the cytosol occurs by a mechanism that depends on endosomal acidification and preserves endosomal integrity, we next addressed a potential involvement of the Golgi complex in the cellular trafficking of cationic CPPs. It was shown recently that uptake of a Tat fusion protein and Tat transactivation activity were sensitive to brefeldin A (30). We tested whether brefeldin A and NDGA (47), compounds that interfere both with the integrity of the Golgi and the trans-Golgi network (TGN), influence the internalization of the cationic CPPs. NDGA is a potent lipoxygenase inhibitor and stimulates the rapid retrograde movement of both Golgi stack and TGN membranes back to the endoplasmic reticulum until both organelles are morphologically absent from cells (47). Thus NDGA can (i) serve as a Golgi-disrupting drug when added prior to the peptide and (ii) provide a means of inducing retrograde transport.

In a first experiment, cells were incubated with brefeldin A or NDGA for 30 min prior to the addition of peptides. For both drugs, the cellular fluorescence of Flu-o-R9 and Flu-o-Antp was reduced significantly. In contrast, for Flu-o-Tat cellular fluorescence was reduced by brefeldin A and slightly enhanced by NDGA (Fig. 6), similar to the effects of bafilomycin A and chloroquine on uptake of Flu-o-Tat (compare Fig. 1, B and C).

**Induction of Retrograde Transport of Fluorescently Labeled CPPs by NDGA—** Since our data suggest that cationic CPPs enter the cytoplasm by means of retrograde transport, we tested whether NDGA is able to affect subcellular localization of Flu-o-Tat in peptide-pulsed cells. In this set of experiments we took advantage of NDGA as an inducer of retrograde transport. HeLa cells were pulsed with Flu-o-Tat for 2 h followed by 3 h of incubation with NDGA in the cell culture medium (Fig. 7). HeLa cells incubated with medium alone during this period exhibited only a vesicular fluorescence, whereas in HeLa cells incubated with NDGA-containing medium a cytoplasmic fluorescence was clearly visible (Fig. 7, C and G). To exclude that this change in cellular distribution was due to a disruption of endosomal compartments by NDGA, cells were co-incubated with AlexaFluor 647-dextran. In contrast to the fluorescein-labeled CPP, NDGA did not affect the localization of the dextran (Fig. 7, B and F), indicating that the CPPs reach a compartment distinct from endosomal compartments and functionally coupled to the cytosol. Similar data were obtained for the Flu-o-Antp peptide.

**Co-localization of Flu-o-Tat with Golgi-specific Fluorescent Probes—** To provide further evidence for the participation of the Golgi complex in the trafficking of cationic CPPs we finally probed for co-localization of Flu-o-Tat with Golgi membranes. The distortion of the distribution of cationic peptides by fixation prohibited an analysis of the co-localization with Golgi-resident proteins by immunofluorescence. Instead cells were incubated with the cell-permeable Golgi tracer Bodipy ceramide. In MC57 cells (Fig. 8, A–D) vesicular peptide fluorescence partially co-localized with Golgi staining; albeit intense peptide staining localized to less intensely stained regions of the Golgi complex. In HeLa cells (Fig. 8, E–H) peptide fluorescence pres-
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**FIG. 3.** Pulse/chase experiments. MC57 cells were incubated with Fluo-Antp (1 μM), Fluo-R9 (1 μM), or Fluo-Tat (5 μM) for 2 h. Internalization was monitored by flow cytometry (P, pulse). For the chase value, aliquots of peptide-loaded cells were washed and incubated with peptide-free medium for an additional 3 h. The remaining cellular fluorescence was determined by flow cytometry (Ch, chase). The Fluo-Tat peptide was applied in a 5-fold higher concentration to compensate for the less efficient intracellular accumulation of this peptide. Each condition was tested in duplicate; error bars represent the absolute deviations from the mean value (A). MC57 cells were incubated ±30 μM Fluo-Tat for 2 h. After 2 h the cells were detached, washed, resuspended in serum-free medium, and incubated for an additional 3 h. The supernatants were desalted, concentrated, and subjected to MALDI-TOF MS analysis (B). Fluo-Tat-derived signals are highlighted with an asterisk. Calculated [M + H]+ were: Fluo-Y-OH, 540.5 Da; Fluo-YG-OH, 597.6 Da; Fluo-YGR-OH, 753.7 Da; Fluo-YGRK-OH, 881.9 Da; Fluo-YGRKK-OH, 1010.1 Da; Fluo-YGRKKR-OH, 1166.3 Da. a.u., arbitrary units.

ent in vesicular structures partially co-localized with the cellular regions of Bodipy ceramide staining. Similar results were obtained for the Fluo-Antp peptide.

**DISCUSSION**

Carboxyfluorescein-labeled CPPs in combination with inhibitors of endocytosis and Golgi-disrupting agents were used to elucidate the intracellular fate of cell-penetrating peptides. Artifacts of cellular peptide distribution induced by fixation were avoided by conducting all experiments with fluorescein-labeled peptides in living cells. Fluorescein was selected as a reporter group because of minimum impact on the cellular fluorescence characteristics when compared to other fluorescent dyes. A vesicular staining could be observed for carboxytetramethylrhodamine-labeled peptides lacking any CPP motif. For CPPs labeled with the Cy5-like indocyanine dye S0387 a patchy fluorescence at the plasma membrane was observed (48). Moreover large collections of fluorescein-labeled CPPs can be generated in high yield and purity carrying the reporter group at a defined position in the peptide (33, 48). These advantages of fluorescein outweigh the limitations imposed by the photophysical characteristics of this dye, i.e. concentration quenching (46) and pH-dependence of its fluorescein characteristics. We addressed these potential drawbacks by determining cellular uptake in whole cell lysates. The discrepancies for uptake efficiencies derived from flow cytometry and fluorescence spectroscopy underline the need for such complementary biochemical analyses. This circumstance needs to be considered if quantitative information about molecules entrapped in intracellular vesicles is to be obtained.

MC57 fibrosarcoma cells incubated with Fluo-Tat for 2 h showed a vesicular fluorescence in accordance with the study by Richard et al. (29). In contrast, both Fluo-R9 and Fluo-Antp also exhibited a homogeneous cytoplasmic and nuclear fluorescence. This observation is indicative of the escape of these latter two CPPs from endocytic compartments and accumulation in the cytoplasm. The basis for the failure to detect Tat in the cytosol was clarified by pulse/chase experiments and analysis of chase supernatants by mass spectrometry. In contrast to Fluo-R9 and Fluo-Antp, after a 3-h chase period in the absence of peptide, the cellular fluorescence for Fluo-Tat decreased by more than 90% of the value after the peptide pulse. Exit of internalized peptides from the cells into the culture medium has always been reported for amphiphatic CPPs (42). However, the MALDI MS analysis of the supernatant of Fluo-Tat-pulsed MC57 cells provided a different explanation. In the supernatant only carboxyfluorescein-labeled fragments of Fluo-Tat carrying 1–6 amino acids could be detected. The poor cellular retention of the Fluo-Tat peptide in MC57 cells therefore likely reflects a high susceptibility to intracellular proteases and the ability of the generated fragments to exit the cells.

The effective retention of Fluo-R9 and Fluo-Antp inside the cells demonstrates that this attribute is sequence- and dye-specific. Moreover the sequence-dependent retention of cellular fluorescence is indicative of intact peptides leaving the endocytic compartment. Given the detection of proteolytic Fluo-Tat fragments in the supernatant of Fluo-Tat-pulsed MC57 cells, the efficient retention of fluorescence for the Fluo-R9 and Fluo-Antp peptides after the 3-h chase period can as well be explained by a preservation of the structural integrity of peptides reaching the cytosol despite a potential to encounter endolysosomal proteases. Using MALDI MS analysis (36) we were able to demonstrate the intracellular integrity of the Fluo-Antp peptide. Apart from this observation the Fluo-R9 peptide exhibits a nuclear enrichment of fluorescence in MC57 cells in the absence of bafilomycin A1 (see Fig. 2C), which further supports the notion of intact peptide entering the cytosol. Highly basic peptide stretches are known to serve as membrane was observed (48). Moreover large collections of fluorescein-labeled CPPs can be generated in high yield and purity carrying the reporter group at a defined position in the peptide (33, 48). These advantages of fluorescein outweigh the limitations imposed by the photophysical characteristics of this dye, i.e. concentration quenching (46) and pH-dependence of its fluorescein characteristics. We addressed these potential drawbacks by determining cellular uptake in whole cell lysates. The discrepancies for uptake efficiencies derived from flow cytometry and fluorescence spectroscopy underline the need for such complementary biochemical analyses. This circumstance needs to be considered if quantitative information about molecules entrapped in intracellular vesicles is to be obtained.

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*R. Fischer and R. Brock, unpublished observations.*
**FIG. 4.** Preservation of endosomal integrity in the presence of Fluo-Antp and intracellular stability of Fluo-Antp. MC57 cells were incubated with serum-free medium containing 5 μM AlexaFluor 647-dextran alone (A–D) or a combination of 5 μM AlexaFluor 647-dextran and 1 μM Fluo-Antp (E–H) for 2 h, washed, and analyzed by multichannel confocal laser scanning microscopy. A and E show transmission pictures, B and F show the AlexaFluor 647-dextran fluorescence, and D and H show the superposition of both fluorescence channels. I, MC57 cells were incubated ±15 μM Fluo-Antp for 2 h. After 2 h the cells were trypsinized, washed, and lysed. The lysates were desalted, concentrated, and subjected to MALDI-TOF MS analysis. Fluo-Antp-derived signals are highlighted with an asterisk. Calculated [M + H]+ were: Fluo-R-OH, 533.5 Da; Fluo-RQIKIWFQNRRMKWKK-NH2, 2605.1 Da. **a.u.**, arbitrary units.

**FIG. 5.** Cellular distribution and uptake of CPPs in different cell lines. MC57 (A, upper panels) and HeLa (A, center panels) cells were incubated with serum-free medium containing Fluo-Antp peptide (1 μM for 2 h) and then analyzed by confocal laser scanning microscopy in the presence of peptide in the medium. Fluorescence images are shown in the left panels; transmission images are shown in the right panels. To exclude incubation conditions as the source of the different peptide distributions, both cell lines were co-cultivated and co-incubated with the Fluo-Antp peptide (A, lower panels). Arrows indicate MC57 cells as judged by cell morphology. MC57 and HeLa cells were incubated with 4 μM Fluo-Antp for 2 h. Bafilomycin A1 (300 nM) (+) was added 30 min prior to the peptide, and internalization was either determined by flow cytometry (B) or by measuring fluorescein concentrations in whole cell lysates (C). The fluorescence present in the cell lysates was normalized to the protein content to correct for differences in cell number and cell size. **a.u.**, arbitrary units.
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of the basis for the different stabilities of Tat and the other two cationic CPPs and enable a rational design of peptides with increased stability.

Since endocytosis and endosomal acidification are required for the lysosomal escape of complexes consisting of the cationic polymer polyethylenimine and ribozymes (50), we reasoned that cationic CPPs may leave the endosomes via a similar mechanism. To assess the role of endocytosis for the uptake of CPPs, cells were incubated with inhibitors of endocytosis and endosomal acidification. To exclude inhibitor-dependent artifacts on cellular uptake, experiments were conducted with wortmannin, chloroquine, and bafilomycin A1 that interfere with endocytosis by different mechanisms. For Fluo-Antp and Fluo-R9 all three inhibitors led to a decrease of cellular fluorescence. The increase of cellular fluorescence for Fluo-Tat in the presence of chloroquine and bafilomycin A1 can be explained by the retention of this peptide inside the endosomal compartment thereby inhibiting the rapid exit of its fragments from the cytosol. Together with the microscopy data obtained, it can be concluded that endosomal acidification is required for the delivery of cationic CPPs to the cytoplasm.

Since endocytosis is a well regulated pathway (51), we asked whether a cell type dependence could also be observed for the localization of CPPs on the subcellular level. Co-cultivation and co-incubation of MC57 fibrosarcoma cells and HeLa cells with the Fluo-Antp or the Fluo-R9 peptide revealed dramatic differences in subcellular distribution. At 1 μM peptide concentration in HeLa cells Fluo-Antp and Fluo-R9 only showed vesicular fluorescence. Almost no cytoplasmic fluorescence could be detected. Comparison of net uptake of both cell types excluded that the vesicular localization of both peptides in HeLa cells resulted from a reduced uptake compared with MC57 cells. Bafilomycin A1 reduced the net uptake in both cell lines significantly, consistent with a similar uptake mechanism but a different intracellular routing after endosomal acidification. The absence of cytoplasmic fluorescence for HeLa cells in the presence of equal amounts of extracellular peptides clearly illustrates that a comparison of biological activities of CPP constructs in different cell types needs to take differences in the subcellular distribution into account.

Next the mechanism by which CPPs leave the endocytic pathway was addressed. Using acidic compartment-specific probes, such as Lysotracker, we tested whether the fluorescein-labeled CPPs reach late endosomes or lysosomes. However, only little or no co-localization was observed for all three CPPs. To rule out that the pH-dependent fluorescence properties of fluorescein impaired these co-localization studies, we also performed these experiments with penetratin labeled with the indocyanine dye S0387 (48), yielding the same results. These data imply that the cationic CPPs leave the endocytic compartment at a rather early stage of endosomal maturation.

To assess whether entry into the cytosol involves endosome disruption, cells were co-incubated with CPPs and a fluores-
cently labeled high molecular weight dextran. However, the presence of the CPP did not affect the distribution of the dextran. In addition, the low cytotoxicity of the CPPs (22), in contrast to agents disrupting endosomes, further supports a mechanism of entry that maintains endosomal integrity.

To this point our observations suggested a mechanism of cellular import that occurs via endocytosis and subsequent translocation of the CPPs via an acidification-dependent passage through endosomal membranes. Such a mechanism would be in line with earlier experiments demonstrating that the penetratin peptide is able to cross a pure lipid bilayer (26). We therefore asked whether an acidic extracellular pH enabled a direct non-endosomal entry of CPPs from the medium into the cytoplasm. Such a pH-dependent direct passage through the plasma membrane was observed for

![Clostridium botulinum C2 toxin](image_url) . This toxin is normally taken up by endocytosis and requires oligomerization and endosomal acidification for release into the cytosol (52). Therefore MC57 cells were briefly pulsed with peptide and then incubated with physiological citrate buffer of various acidic pH values (pH 5–7). However, during the incubation time tolerated by the cells, no cytosolic fluorescence was observed (data not shown).

The failure to obtain evidence for a direct passage of the CPPs from the endosomal compartment into the cytoplasm prompted us to address an involvement of retrograde transport on cellular trafficking. It was shown that brefeldin A affects uptake and cytosolic biological activity of Tat-green fluorescent protein fusion proteins (30). For this reason, we investigated the effects of brefeldin A and NDGA on the net uptake of the fluorescently labeled CPPs in MC57 and HeLa cells. When NDGA or brefeldin A were applied to the cells prior to the addition of the peptide, the net uptake of Fluo-R9 and Fluo-Antp was reduced for both agents as observed by flow cytometry. However, cellular fluorescence of Fluo-Tat-treated MC57 cells was slightly enhanced by NDGA (see Fig. 6B). This result implies that for Fluo-Tat disruption of retrograde trafficking traps this peptide in the endosomal compartment thereby preventing its entry into the cytosol and rapid cellular exit of its fragments (as shown in Fig. 3). This circumstance led to an enhanced cellular fluorescence in the case of Fluo-Tat in the

![TABLE II: Arginine-rich motifs in plant and bacterial toxins](image_url) . Primary structures of the arginine-rich motifs with high sequence homology to the HIV-1 Tat peptide are shown. Sequence homologies were identified by visual inspection of the respective proteins. The arginine-rich sequence of the HIV-1 Tat protein is included as entry 5. Sequence information was obtained from www.ncbi.nlm.nih.gov/entrez/query.fcgi.

| Entry | Protein                     | Organism                        | NCBI accession number | Protein function                        | Sequence localization | Amino acid sequence |
|-------|-----------------------------|---------------------------------|-----------------------|-----------------------------------------|-----------------------|--------------------|
| 1     | Verotoxin-2 variant, subunit A | *Escherichia coli*              | AAP37403              | Cleavage of glycoside bond within 28 S rRNA | 192–201               | RFRQIQREFR         |
| 2     | Shiga toxin, subunit A       | *Shigella dysenteriae*          | AAF28121              | Cleavage of glycoside bond within 28 S rRNA | 192–201               | RFRQIQREFR         |
| 3     | Cholera enterotoxin, A chain | *Vibrio cholerae*               | P01555                | ADP-ribosylation of G,α                   | 159–166               | RNRYGDRR           |
| 4     | Ricin, A chain               | *Ricinus communis*              | 2AA1A                 | Cleavage of glycoside bond within 28 S rRNA | 189–207               | RTIRYRRR           |
| 5     | Tat, transactivating regulatory protein | Human immunodeficiency virus 1 | P04613                | Transcriptional regulation                | 49–57                 | RKKRRQRRR          |

* National Center for Biotechnology Information.

![Fig. 9: Dissection of intracellular trafficking of cationic CPPs by small molecule drugs](image_url) . A, summary of the results of CPP trafficking along the endosomal and retrograde pathway obtained using small molecule inhibitors. B and C, effect of NDGA on the localization and trafficking of cationic CPPs upon incubation of cells with the compound before addition of peptide (B) and addition after a 2-h pulse with peptide that allowed the peptide to enter the Golgi and trans-Golgi network (C). Dark shades indicate an accumulation of peptide in the respective compartment. ER, endoplasmic reticulum.
presence of NDGA. In the case of Fluo-R9 and Fluo-Antp the absence of TGN and the Golgi complex led to reduced uptake. In a second series of experiments we utilized the ability of NDGA to induce retrograde transport in cells pulsed with Fluo-Tat or Fluo-Antp for 2 h. The observed increase of cytoplasmic Tat fluorescence in peptide-pulsed and subsequently NDGA-treated HeLa cells strongly supports an involvement of the Golgi apparatus or the TGN in the uptake of Tat peptides (see Fig. 7). Essentially the same result was obtained for Fluo-Antp (data not shown). The brefeldin A-sensitive transactivation activity of Tat-green fluorescent protein fusion proteins (30) also supports this hypothesis. The influence of furin, a TGN-resident protease, on the processing of Tat-major histocompatibility complex class I constructs (53) provides further evidence for a passage of Tat peptides through the TGN.

Finally partial co-localization of Fluo-Tat-derived vesicular structures with the Golgi marker Bodipy ceramide was observed in HeLa cells. Similar co-localization data were obtained for Fluo-Antp (data not shown). The inability to detect bulk quantities of Fluo-Tat and Fluo-Antp in the Golgi complex in MC57 cells might be due to a rapid passage through this organelle. The plant toxin ricin, for example, which is known to traverse the Golgi complex and the endoplasmic reticulum, has never been visualized by microscopy in the endoplasmic reticulum (54). For this reason it was initially suggested that the ricin A chain translocates from the TGN directly to the cytosol (55).

For the cationic CPPs, the apparent involvement of the Golgi complex bears a striking resemblance to features of some bacterial toxins. The major histocompatibility complex class I constructs are processed proteolytically by furin, a TGN-resident protease, on the processing of Tat-major histocompatibility complex class I constructs (53) provides further evidence for a passage of Tat peptides through the TGN.

REFERENCES
1. Stephens, D. J., and Pepperkok, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4285–4288
2. Langel, U. (2002) Cell Penetrating Peptides: Processes and Applications, CRC Press, Boca Raton, FL
3. Fischer, P. M., Krause, E., and Lane, D. P. (2001) Bioconjug. Chem. 12, 825–841
4. Schütte-Redelmeier, M.-P., Gournier, H., Garcia-Pons, F., Mousa, M., Joliot, A. H., Volvich, M., Prochiantz, A., and Lemmonier, F. A. (1996) J. Immunol. 157, 633
5. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) Science 285, 1569–1572
6. Prochiantz, A. (1999) Curr. Opin. Neurobiol. 6, 629–634
7. Hawiger, J. (1999) Curr. Opin. Chem. Biol. 3, 89–94
8. Rojas, M., Donahue, J. P., Tan, Z., and Lin, Y. Z. (1998) Nat. Biotechnol. 16, 370–375
9. Astrab-Fisher, A., Sergeev, D., Fischer, M., Shaw, B. R., and Juliano, R. I. (2002) Pharm. Res. 19, 744–754
10. Singh, D., Bolland, S. K., Kawamura, K., and Gariepy, J. (1999) Bioconjug. Chem. 10, 745–754
11. Pooga, M., Soomets, U., Hallbrink, M., Vainla, A., Saar, K., Rezaei, K., Kahl, U., Hao, J.-X., Xu, X.-J., Wiesenfeld-Hallin, Z., Hoftek, H., Bartfai, T., and Langel, U. (1998) Nat. Biotechnol. 16, 857–861
12. Lewin, M., Carlesso, N., Tung, C.-H., Tang, X.-W., Cory, D., Scadden, D. T., and Weisell, R. (2000) Nat. Biotechnol. 18, 410–414
13. Tseng, L., Derosi, D., Chassaing, G., Llibre, B., Kubes, M., Jordan, P., Ch neutweiss, H., Godement, P., and Prochiantz, A. (1995) J. Neurosci. 15, 7158–7167
14. Niznik, K., Sawadzidskal, S., Fruman, D. A., Lai, J., Songyang, Z., Burakoff, S. J., Yaffe, M. B., and Cantley, L. C. (2000) Mol. Cell 6, 969–974
15. Lin, Y.-Z., Yao, S. Y., Yeach, R. A., Torgerson, T. R., and Hawiger, J. (1995) J. Biol. Chem. 270, 14255–14258
16. Horeau, T., Barton, G. M., and Medohit, R. (2001) Nat. Immunol. 2, 835–841
17. Pietersz, G. A., Li, W., and Apostolopoulos, V. (2001) Vaccine 19, 1397–1405
18. Jung, G., and Beck-Sickinger, A. G. (1992) Angew. Chem. Int. Ed. Engl. 31, 367–383
19. Liu, Y.-Y., Timmons, S., Lin, Y.-Z., and Hawiger, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11819–11824
20. Wang, R.-F., and Wang, H. Y. (2002) Nat. Biotechnol. 20, 149–154
21. Derosi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) J. Biol. Chem. 269, 10444–10450
22. Vives, E., Brodin, P., and Leblou, B. (1997) J. Biol. Chem. 272, 16010–16017
23. Prochiantz, A. (2000) Int. J. Pharm. 245, 1–7
24. Futaki, H., Futaki, S., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugura, Y. (2001) J. Biol. Chem. 276, 5836–5840
25. Futaki, S., Futaki, H., Niwa, M., Tanaka, S., Ueda, K., and Sugura, Y. (2001) J. Biol. Chem. 277, 2437–2443
26. Thoren, P. E. G., Persson, D., Karlsson, M., and Norden, B. (2000) FEBS Lett. 492, 265–268
27. Persson, D., Thoren, P. E. G., Herner, M., Lincoln, P., and Norden, B. (2003) Biochemistry 42, 421–429
28. Derosi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G., and Prochiantz, A. (1996) J. Biol. Chem. 271, 18188–18193
29. Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chassaing, G., and Prochiantz, A. (1994) J. Biol. Chem. 269, 10396–10402
30. Chassaing, G., and Prochiantz, A. (1994) J. Biol. Chem. 270, 14255–14258
31. Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F., and Gait, M. J. (1994) J. Biol. Chem. 269, 14257–14268
32. Consalez, G., Garcia-Echevarria, C., Huttunen, J. K., and Lebleu, B. (1997) J. Biol. Chem. 272, 35109–35114
33. Fischer, R., Roder, M., Jung, G., and Brock, R. (2003) Bioconjug. Chem. 14, 561–569
34. Sarin, V. K., Kent, S. B., Tam, J. P., and Merrifield, R. B. (1981) Anal. Biochem. 117, 147–157
35. Hosaka, Y., Yasuda, Y., Sereburi, O., Moran, M. G., and Fukai, K. (1996) J. Virol. 70, 1113–1118
36. Elmquist, A., and Langel, U. (2003) Biochem. Biophys. Res. Commun. 302, 387–393
37. Simonsson, A., Lippe, R., Christoforidis, S., Gaulier, J. M., Brech, A., Callaghan, J., Tob, E.-H., Murphy, C., Zerial, M., and Stenmark, H. (1998) Nature 394, 494–498
38. de Duve, C., DeBarsy, T., Polle, A., Tricot, A., Tulkens, P., and Van Hooff, J. F. (1974) Biochem. Pharmacol. 23, 2495–2503
39. Keski, K., Ukkonen, P. S., Pratt, E. J., and Kabat, D. (1999) J. Biol. Chem. 274, 23499–23507
40. Bowman, E. J., Siebers, A., and Kickhoefer, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7972–7976
41. Clague, M. J., Urbe, S., Aniento, F., and Grunenberg, J. (1994) J. Biol. Chem. 269, 6043–6049
42. Fischer, R., Waizenegger, T., Köhler, K., and Brock, R. (2002) Biochim. Biophys. Acta 1564, 365–374
Routing of Cationic Cell-penetrating Peptides

49. Jans, D. A., Xiao, C.-Y., and Lam, M. H. C. (2000) Bioessays 22, 532–544
50. Merdan, T., Kunath, K., Fischer, D., Kupecek, J., and Kissel, T. (2002) Pharm. Res. 19, 140–147
51. Gruenberg, J. (2001) Nat. Rev. Mol. Cell. Biol. 2, 721–730
52. Barth, H., Blobker, D., Behlke, J., Bergema-Schutter, W., Brisson, A., Benz, R., and Aktories, K. (2000) J. Biol. Chem. 275, 18704–18711
53. Lu, J., Wettstein, P. J., Higashimoto, Y., Appella, E., and Celis, E. (2001) J. Immunol. 166, 7063–7071
54. Sandvig, K., and van Deurs, B. (2002) FEBS Lett. 529, 49–53
55. van Deurs, B., Sandvig, K., Petersen, O. W., Olesen, S., Simons, K., and Griffiths, G. (1988) J. Cell Biol. 106, 253–267
56. Sandvig, K., and van Deurs, B. (2000) EMBO J. 19, 5943–5956
57. Lord, J. M., and Roberts, L. M. (1998) J. Cell Biol. 140, 733–736
58. Garred, O., van Deurs, B., and Sandvig, K. (1995) J. Biol. Chem. 270, 10817–10821
59. Sandoval, R. M., and Molitoris, B. A. (2004) Am. J. Physiol., in press