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No entry for TAT(44–57) into liposomes and intact MDCK cells: novel approach to study membrane permeation of cell-penetrating peptides

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Abstract

Cell penetrating peptides (CPPs) have been postulated to carry macromolecules across cell plasma membranes without the need of receptors, transporters, endocytosis or any energy-consuming mechanism.

We developed an assay to study lipid bilayer permeation of CPPs. HIV-1 TAT peptides were conjugated to *N*-(4-carboxy-3-hydroxyphenyl)maleimide (SAM) and incubated with Tb^{3+}-containing liposomes. Upon chelation of Tb^{3+} by an aromatic carboxylic acid, the fluorescence of Tb^{3+} increases many fold. The CPP TAT(44–57)-SAM and TAT(37–53)-SAM, as a negative control, were unable to enter liposomes consisting of phosphatidylcholine (PC) or a mix of PC, negatively charged lipids and cholesterol.

In parallel, cell entry of fluorescein-labeled TAT peptides was studied using confocal laser scanning microscopy (CLSM). TAT(44–57)-fluorescein did not enter Madin Darby canine kidney (MDCK) cells with intact plasma membranes but accumulated at their basal side. Only cells with impaired plasma membranes, as identified by nuclear staining with ethidium homodimer-1 (EthD-1), showed accumulation of TAT(44–57).

Our findings change the perspectives of the potential use of TAT peptides as carriers for intracellular targeting. SAM- and fluorescein-labeled TAT(44–57) cannot penetrate lipid bilayers and intact plasma membranes of MDCK cells, respectively.

Keywords: Cell-penetrating peptide; Ethidium homodimer-1; Liposome; Membrane permeation; TAT peptide; MDCK cell

1. Introduction

The discovery of cell-penetrating peptides (CPPs) that are capable to carry macromolecules across cell plasma membranes opened new perspectives for the intracellular targeting of peptides [1], enzymes [2,3], oligonucleotides [4] and even larger structures such as liposomes [5] or nanoparticles [6]. During the last few years, several CPPs have been described. Certain CPPs seem to permeate cell plasma membranes without the need of transporters, receptors or endocytosis. Hydrophobicity is not a requirement. The exact mechanism of cell entry is not yet identified and is presumably different for the diverse types of peptides. Reviews by Prochiantz [7], Lindgren et al. [8] and Fischer et al. [9] give a comprehensive overview.

TAT peptides, a subclass of CPPs, are frequently applied for intracellular targeting of macromolecules in in vitro and in vivo assays. They are derived from the TAT protein, a 86-residue transcriptional regulator of the HIV-1 virus. The protein that contains a nuclear localization sequence has been reported to traverse cell membranes. According to Tyagi et al. [10], cell entry of the full-length TAT protein follows binding to cell surface heparan sulfate proteoglycans. Liu et al. [11] found that cell entry is mediated by low-density lipoprotein receptor-related protein. It has been shown that the basic sequence TAT(48–57) is sufficient for membrane translocation [12]. TAT peptides containing

Abbreviations: CLSM, confocal laser scanning microscopy; CPP, cell-penetrating peptide; EBSS, Earl's balanced salt solution; EDTA, ethylene-di-amine-tetra-acetic acid; EthD-1, ethidium homodimer-1; FM, fluorescein-5-maleimide; GM1, monosialoganglioside; Hac, acetic acid; MDCK, Madin Darby canine kidney; MOPS, 3-(*N*-morpholino)propane sulfonic acid; PBS, phosphate buffered saline; PC, phosphatidylcholine; SA, salicylic acid; SAM, *N*-(4-carboxy-3-hydroxyphenyl)maleimide

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this sequence appear to carry proteins and fluorescent molecules into cells in vivo or in culture [3,13,14]. According to previous reports, unlike full-length TAT protein, such peptide constructs permeate cell plasma membranes independently of classical cell entry mechanisms [9,15]. The high content of basic amino acids in TAT(48–57) and in other CPPs of this type are thought to constitute a transduction domain.

Vivès et al. [13] suggested an invagination of the plasma membrane upon binding of TAT peptides to negative charges of the lipids with the subsequent release of the peptide into the cytoplasm. Prochiantz [7] put forward the hypothesis that the membrane translocation peptides derived from the *Dorsophila* transcription factor Antennapedia, i.e. penetratins, induce the formation of inverted micelles in biological membranes. Although there is some evidence from a study with giant liposomes that penetratin crosses lipid membranes consisting of a mix of naturally occurring lipids [16], there is no clear proof of translocation of such peptide conjugates across lipid bilayers.

Today, an assay is still lacking which would allow to unambiguously study the translocation of such peptides across lipid bilayers. Such an assay would give useful information on the permeation mechanism and show whether indeed no receptor, transporter or other protein-dependent mechanism is needed for translocation. It would facilitate the search for new CPPs and membrane characteristics favoring transduction.

In this study, we developed an assay that makes it possible to clearly analyze the entry of a peptide conjugate into the liposomal lumen. For a start, we tested two TAT peptides: TAT(44–57), which is known for its membrane translocation characteristics [14] and TAT(37–53), which contains less positive charges and is not able to enter cells even at high concentrations [13]. Both peptides were conjugated with *N*-(4-carboxy-3-hydroxyphenyl)maleimide (SAM) and incubated with Tb^{3+}-containing liposomes. Aromatic carboxylic acids such as conjugated SAM increase the fluorescence of Tb^{3+} at 545 nm many fold upon chelation when excited at a wavelength absorbed by the aromatic system of the complex [17]. This made it possible to investigate the permeation capacity of TAT peptide conjugates across lipid membranes of different compositions.

In parallel, we studied cell entry of the fluorescein-labeled peptides on Madin Darby canine kidney (MDCK) cell cultures using confocal laser scanning microscopy (CLSM). The integrity of the plasma membranes was checked with ethidium homodimer-1 (EthD-1), a stain used for viability tests. EthD-1 is not able to cross integer plasma membranes and displays fluorescence upon binding to nucleic acids [18]. We could not detect any permeation of conjugated TAT peptide across lipid bilayers. Entry into MDCK cells was only seen in cells that were also permeable for EthD-1.

2. Materials and methods

2.1. Materials

The peptides Cys-TAT(44–57) (H_2N-CGISYGRKKRRQRRR-$CONH_2$) and CYS-TAT(37–53) (H_2N-CFITKALGISYGRKKRR-$CONH_2$) were purchased from Eurogentec (Belgium). Peptides were >85% pure and identity was checked by HPLC-MS by the supplier. Egg phosphatidylcholine (PC, grade I) and other phospholipids were delivered by Lipid Products (Nutfield, UK) and bovine brain monosialoganglioside G_{M1} by Fluka. Cholesterol and SAM were obtained from Sigma, fluorescein-5-maleimide (FM), Hoechst 33342 and EthD-1 from Molecular Probes. Terbium(III) chloride hexahydrate was purchased from Aldrich. Methanol for liposome preparation was of HPLC quality. All other compounds were of analytical grade.

2.2. Peptide labeling with SAM

Peptides were incubated at a concentration of 10 mM with 8 mM SAM in 0.16 M acetate buffer (Hac) pH 6.0 for 1 h at room temperature. Identity and purity of the conjugated Cys-TAT(44–57)-SAM were confirmed by C-18 reversed-phase HPLC and subsequent MALDI-TOF analysis.

2.3. Peptide labeling with FM

Cys-TAT(44–57) and Cys-TAT(37–53) were labeled with FM as described by Niesner et al. [14]. In brief, 100 μM FM in 10% (v/v) dimethylformamide in PBS pH 7.4 was incubated with 1 mM Cys-TAT for 60 min at room temperature. No residual FM was detectable using HPLC.

2.4. Liposomes

For the preparation of Tb^{3+}-containing liposomes, between 20 and 100 mg lipids were dissolved together with 0.2 mmol terbium(III) chloride in 3 ml methanol in a 250-ml round flask. The solvent was evaporated using a rotavapor and the resulting film of lipid and terbium(III) chloride was kept at <10 mbar for a few hours. The dried film was rehydrated with 2 ml water and the so formed multilamellar liposomes were subjected to five cycles of freeze-thawing using solid CO_2 in ethanol. Unilamellar liposomes were prepared by 10 times extrusion through Nucleopore® polycarbonate membranes (Corning) with 200 nm pore diameter using an extruder from Lipex Biomembranes Inc. (Vancouver, Canada) [19]. External Tb^{3+} was removed on a Sephadex G-25 PD-10 desalting column (Amersham Biosciences). The column was equilibrated with 200 mM NaCl and 1 ml of liposome preparation was loaded and eluted with 200 mM NaCl. The first 0.2 ml of the liposome fraction were discarded, the

following 1 ml collected for fluorescence assays. The passage over the column resulted in a dilution factor of about 2. The average mean sizes were between 160 and 200 nm, polydispersity factors were <0.1 as determined by a Zetasizer 3 (Malvern). Size distributions were unchanged after the fluorescence assays. Extra liposomal Tb^{3+} was negligible for several days when liposomes were stored at 4 °C.

2.5. Concentration of entrapped Tb^{3+}

To estimate the concentration of entrapped Tb^{3+} in the final liposome preparation, lipids were extracted with chloroform. Salicylic acid (SA, 25 μM) was added to the aqueous phase and the pH adjusted to 7.0 with 20 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS). Fluorescence scans were recorded between 520 and 570 nm at λ_{ex} 318 nm while the sample was titrated with ethylenediaminetetraacetic acid (EDTA). The peak at 545 nm disappeared upon displacement of SA from the SA/Tb^{3+} complex by EDTA. The EDTA concentration at complete flattening of the emission spectrum equaled the Tb^{3+} concentration in the sample as confirmed in control experiments. Assuming a spherical shape of the liposomes at a size of 200 nm, the intraliposomal Tb^{3+} concentration was between 5 and 7 mM.

2.6. Fluorescence assays

To study membrane permeation of peptide-SAM or SA, 450 μl of liposome eluate were adjusted to pH 7.0 with 50 μl 200 mM MOPS. Between 1 and 10 μl of peptide conjugate or SA in 0.16 M Hac pH 6.0 were added to give the final concentrations indicated under Results. Fluorescence scans were monitored over time between 520 and 570 nm at λ_{ex} 318 nm. Temperature was kept constant at 20 °C. As a control, 0.16 M Hac pH 6.0 was used instead of the peptide conjugates or SA.

2.7. Data analysis

The increase in fluorescence at 545 nm $\Delta F_{545\ nm}$ was calculated from the emission scans as follows:

$$\Delta F_{545\ nm} = F_{545\ nm} - \frac{F_{530\ nm} + F_{560\ nm}}{2} \quad (1)$$

where F is the fluorescence in arbitrary units at the wavelength indicated by the index.

2.8. Cells

MDCK cells (type II) were grown in Eagle's minimum essential medium with Earl's salts (EMEM) containing 10% fetal calf serum under standard conditions [20].

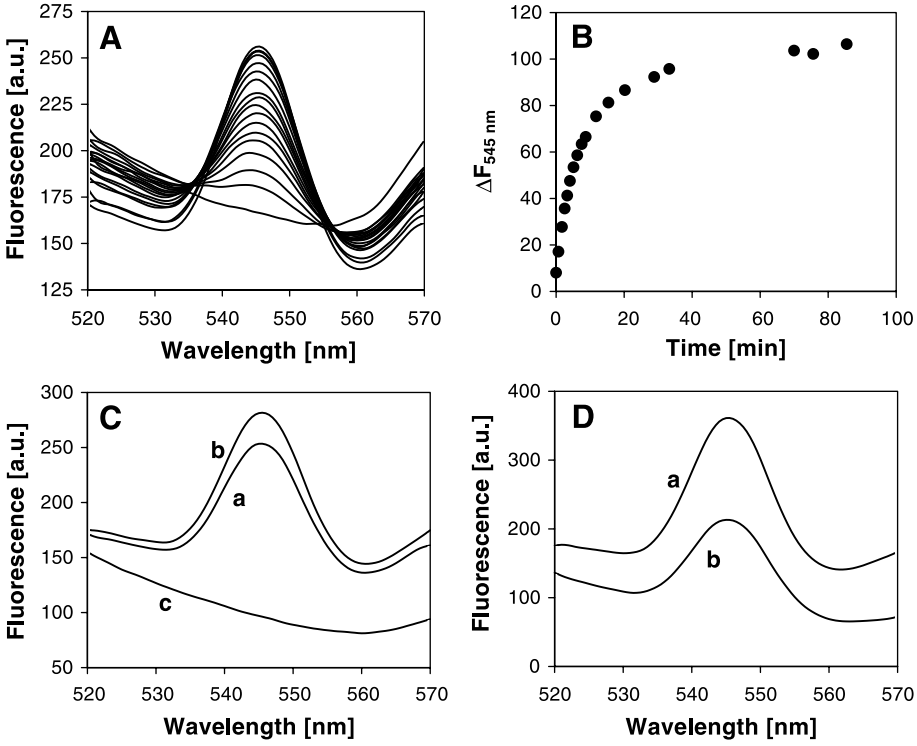

Fig. 1. Entry of SA into Tb^{3+}-containing egg PC liposomes. Tb^{3+}-containing egg PC liposomes were incubated with SA and fluorescence scans were monitored at λ_{ex} 318 nm (details see text). (A) Fluorescence scans before addition of SA (no peak at 545 nm) and at different time points between 0 and 85 min after SA addition. (B) Peak heights $\Delta F_{545\ nm}$ as calculated from the spectra in (A) according to Eq. (1). (C) After incubation of liposomes with SA for 85 min (a), EDTA was added to displace SA from extraliposomal Tb^{3+} (b). After EDTA addition, liposomes were lysed with Triton X-100 (c). (D) After incubation of liposomes with SA for 50 min (a), liposomes were lysed with Triton X-100 in the absence of EDTA (b). a.u., arbitrary units.

2.9. Confocal laser scanning microscopy

MDCK cells grown on glass cover slips for 1 or 2 days (70–80% confluent) were washed twice with Earl's balanced salt solution (EBSS). The cultures were incubated at 37 °C as indicated with EBSS or medium without phenol red and FCS containing peptide-FM or unconjugated FM, 2 μM Hoechst 33342 to stain DNA and/or 4 μM EthD-1 as a probe for plasma membrane integrity. Cells were mounted without fixation and scanned using a Zeiss CLSM 410 inverted microscope [20]. 3D multichannel image processing was performed using the IMARIS software (Bitplane AG, Switzerland).

3. Results

3.1. Permeation of SA and TAT peptide-SAM across lipid bilayers

Peptide-SAM/Tb^{3+} and SA/Tb^{3+} complexes show increased fluorescence at 545 nm when excited at 318 nm as compared to the noncomplexed Tb^{3+}. In order to find out whether TAT(44–57) is able to cross lipid bilayers independently of any receptors or cellular mechanisms, Tb^{3+}-containing liposomes of different lipid compositions were incubated with Cys-TAT(44–57)-SAM and fluorescence scans were monitored over time as described under Materials and methods. To test the assay, liposome entry of SA was measured under the same conditions.

3.2. Fluorescence increase after SA addition to Tb^{3+}-containing liposomes

Fig. 1A shows the fluorescence spectra of a sample containing Tb^{3+}-loaded egg PC liposomes at pH 7.0 at different time points between 0 and 85 min after addition of SA. The lipid concentration was about 5 mg/ml, the overall Tb^{3+} concentration about 0.25 mM and the overall SA concentration 0.05 mM. Fluorescence at 545 nm increased by about 100 arbitrary units reaching a plateau after 1 h. The peak height $\Delta F_{545\ nm}$ was calculated according to Eq. (1) and plotted against time in Fig. 1B.

To confirm the intraliposomal localization of the fluorescent SA/Tb^{3+} complex, EDTA was added at a final concentration of 5 mM. EDTA is a stronger chelator for Tb^{3+} than SA and displaces SA from the complex with Tb^{3+} leading to an extinction of fluorescence at 545 nm [17]. As shown in Fig. 1C, EDTA addition did not lower $\Delta F_{545\ nm}$, indicating that the fluorescence resulted from

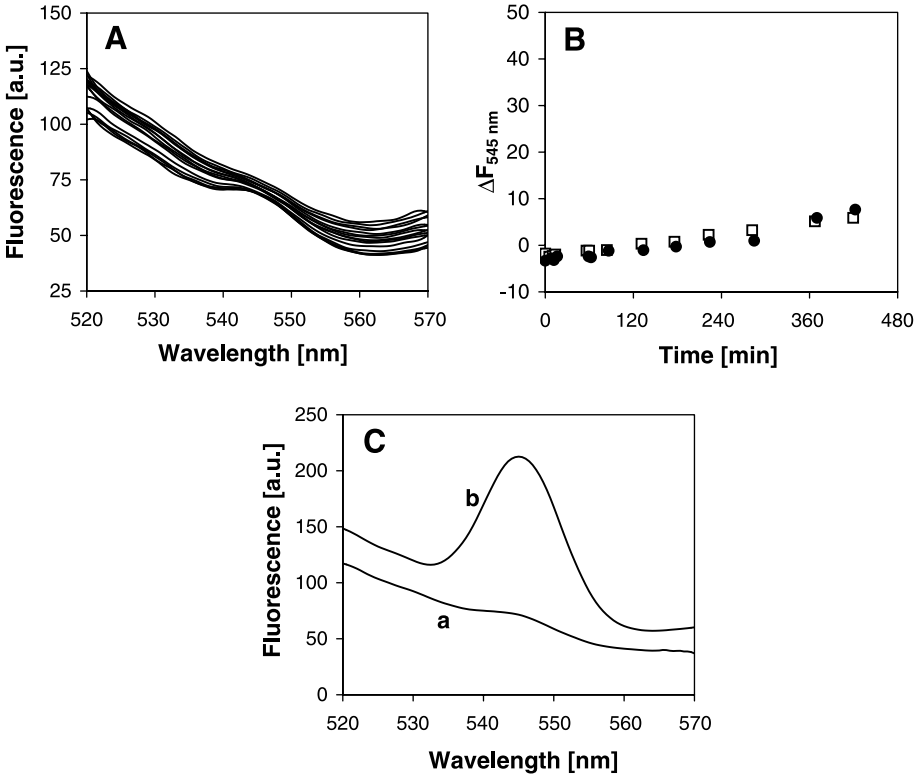

Fig. 2. Ability of Cys-TAT(44–57)-SAM to enter Tb^{3+}-containing liposomes. Tb^{3+}-containing egg PC liposomes were incubated with 0.2 mM Cys-TAT(44–57)-SAM or without peptide-conjugate. Fluorescence scans were monitored at λ_{ex} 318 nm (details see text). (A) Fluorescence scans at different time points between 0 and 420 min after peptide-SAM addition. (B) Peak heights $\Delta F_{545\ nm}$ as calculated from the spectra in (A) according to Eq. (1). Filled circles, control without TAT-SAM; open squares, Cys-TAT(44–57)-SAM. (C) After incubation of liposomes with Cys-TAT(44–57) for 80 min (a), liposomes were lysed with Triton X-100 (b). a.u., arbitrary units.

intraliposomal complexes. Only upon lysis of the liposomes with 1% Triton X-100 did the peak at 545 nm completely disappear in the EDTA-containing sample (Fig. 1C). At this concentration, the detergent has only minor effects on the fluorescence spectra of the Tb^{3+} complexes. Fig. 1D shows the spectra of a similar sample after fluorescence has reached its maximum and after Triton X-100 was added without EDTA. In this case, the spectra are similar before and after lysis of the liposomes.

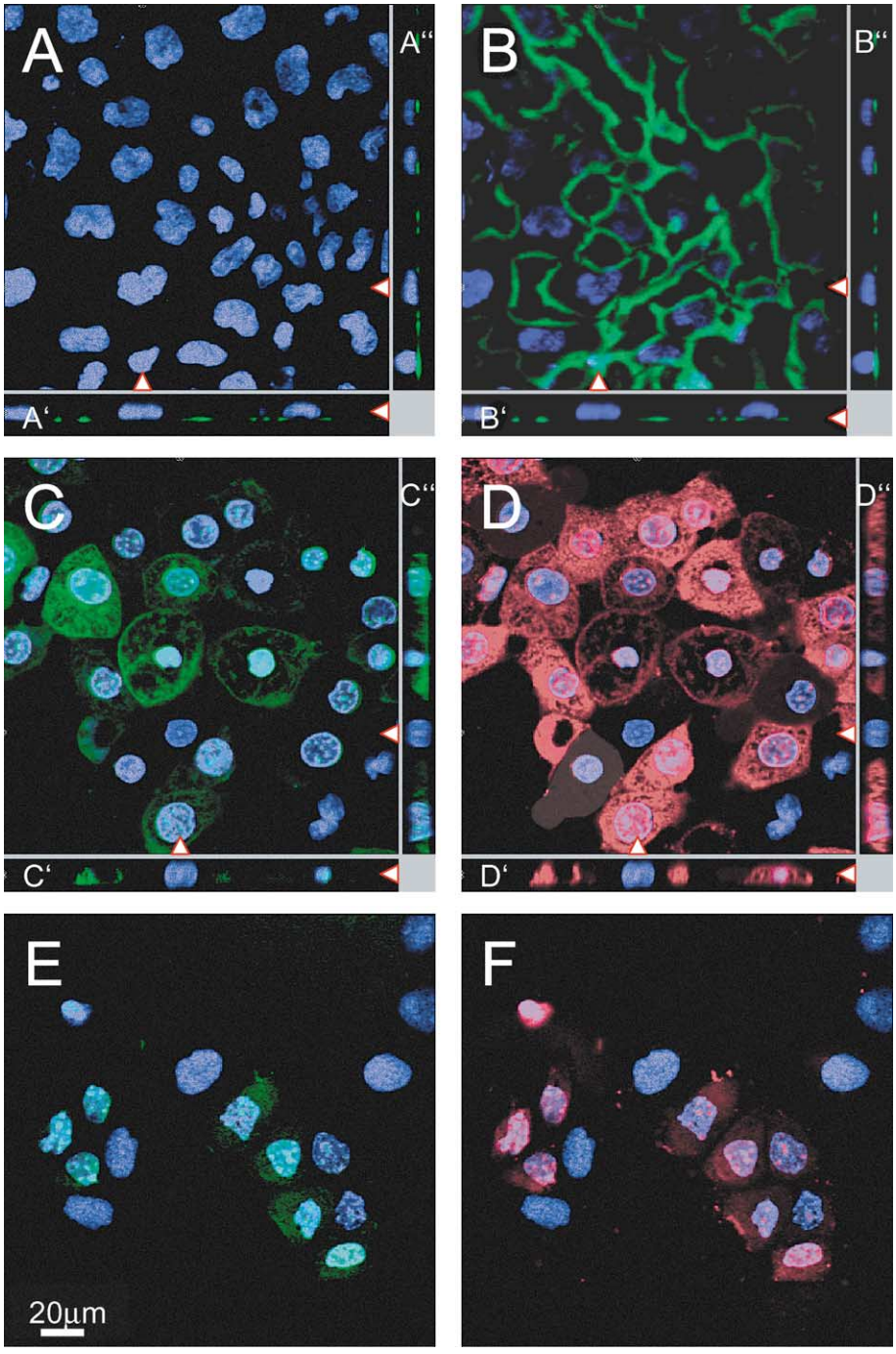

Fig. 3. Cys-TAT(44–57)-FM in a CLSM permeation assay with MDCK cells. MDCK cells grown for 1–2 days on glass cover slips were incubated for 20 min with Cys-TAT(44–57)-FM (green), Hoechst 33342 (blue) and EthD-1 (red) in EBSS at 37 °C. Cells were mounted without washing or fixation. (A–D) 1 μM peptide-FM (10 μM total peptide); (E,F) 0.2 μM peptide-FM (2 μM total peptide). (A) *x*, *y* optical section through the center of the nuclei immediately after mounting and (B) through a lower part of the same cells close to the cover slip. (A′,B′) and (A″,B″) *x*, *z* and *y*, *z* projections. Arrowheads indicate the positions of the projections. (C,E) *x*, *y* optical sections through the center of the nuclei of cells with peptide-FM uptake 15 min after mounting. Co-localization of peptide-FM and Merck 33342 appears in turquoise. (D,F) EthD-1 staining of the respective areas. Co-localization of EthD-1 and Merck 33342 appears as magenta.

3.3. Ability of Cys-TAT(44–57)-SAM to permeate lipid bilayers

Using the same protocol as described for SA, the abilities of Cys-TAT(44–57)-SAM and Cys-TAT(37–53)-SAM to permeate lipid membranes were investigated with liposomes of different lipid compositions. Fig. 2A shows scans over 420 min in a sample containing egg PC liposomes (lipid concentration 25 mg/ml, 0.75 mM Tb^{3+}) after the addition of 0.2 mM Cys-TAT(44–57)-SAM. The respective $\Delta F_{545\ nm}$ values were plotted against time and compared to a control sample without any peptide-SAM or SA in Fig. 2B. No

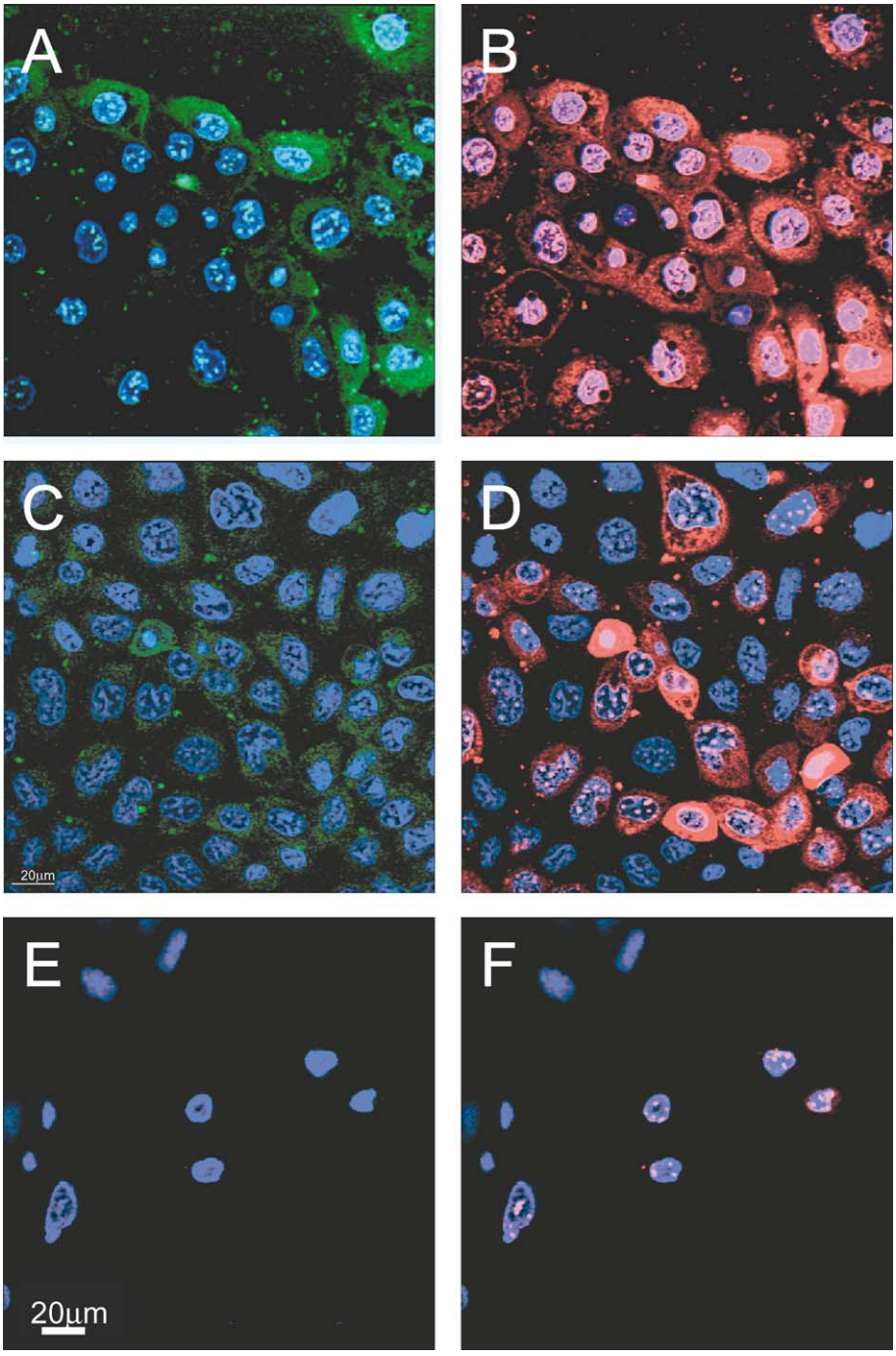

Fig. 4. Cys-TAT(37–53)-FM in a CLSM permeation assay with MDCK cells. Cells were grown for 1–2 days on glass cover slips and incubated with Cys-TAT(37–53)-FM, Merck 33342 and EthD-1 in EBSS for 20 min at 37 °C. Cells were mounted without washing or fixation and x, y scans through the center of the nuclei were taken immediately after mounting. (A,B) 1 μM peptide-FM (10 μM total peptide); (C,D) 0.2 μM peptide-FM (2 μM total peptide). In both cultures, EthD-1 stained >50% of total nuclei. (E,F) Cells were incubated with 1 μM FM, Merck 33342 and EthD-1 for 20 min in EBSS at 37 °C and scanned immediately after mounting. EthD-1 stained < 5% of total nuclei.

significant differences could be found between the $\Delta F_{545\ nm}$ values of the peptide-SAM containing sample and the control sample over time. $\Delta F_{545\ nm}$ increased by about 10 after 7 h due to hydroxylation of Tb^{3+}. Fig. 2C shows the scans of a parallel sample after 80 min incubation with Cys-TAT(44–57)-SAM before and after Triton X-100 addition (1%). Upon liposome lysis, Cys-TAT-SAM was able to complex Tb^{3+} ions, resulting in an increase in $\Delta F_{545\ nm}$ to about 120. Similar findings were made for Cys-TAT(37–53)-SAM in the egg PC liposome system and for Cys-TAT(44–57)-SAM with liposomes containing 20% (mol/mol) cholesterol, 20% phosphatidylserine, 5% oleic acid, 5% phosphatidylinositol or 1% GM1.

From this, we conclude that Cys-TAT(44–57)-SAM is not capable to cross lipid bilayers to a significant extent. All experiments were repeated several times at varying lipid concentrations between 5 and 25 mg/ml and Cys-TAT(44–57)-SAM concentrations between 0.02 and 1 mM with reproducible results. Representative examples are shown.

3.4. Uptake of fluorescein-labeled TAT peptides into MDCK cells

In order to study uptake of TAT peptides into cultured cells using CLSM, Cys-TAT peptides were conjugated with FM as described under Materials and methods. MDCK cells were incubated for 20 min at 37 °C with EBSS containing the labeled Cys-TAT peptides or unconjugated FM as a control together with Hoechst 33342 to stain DNA and EthD-1 to identify cells with impaired plasma membranes. Final total peptide concentrations were either 2 or 10 μM, as indicated, of which 10% were labeled with FM. CLSM scans were taken from preparations without fixation.

No entry of Cys-TAT(44–57)-FM into cells with integer plasma membranes was observed at both concentrations. Only cells permeable for EthD-1 were also permeable for TAT(44–57)-FM. Fig. 3A shows an optical section (x, y) taken through the center of the nuclei of a cell monolayer incubated with 1 μM Cys-TAT(44–57)-FM (10 μM total

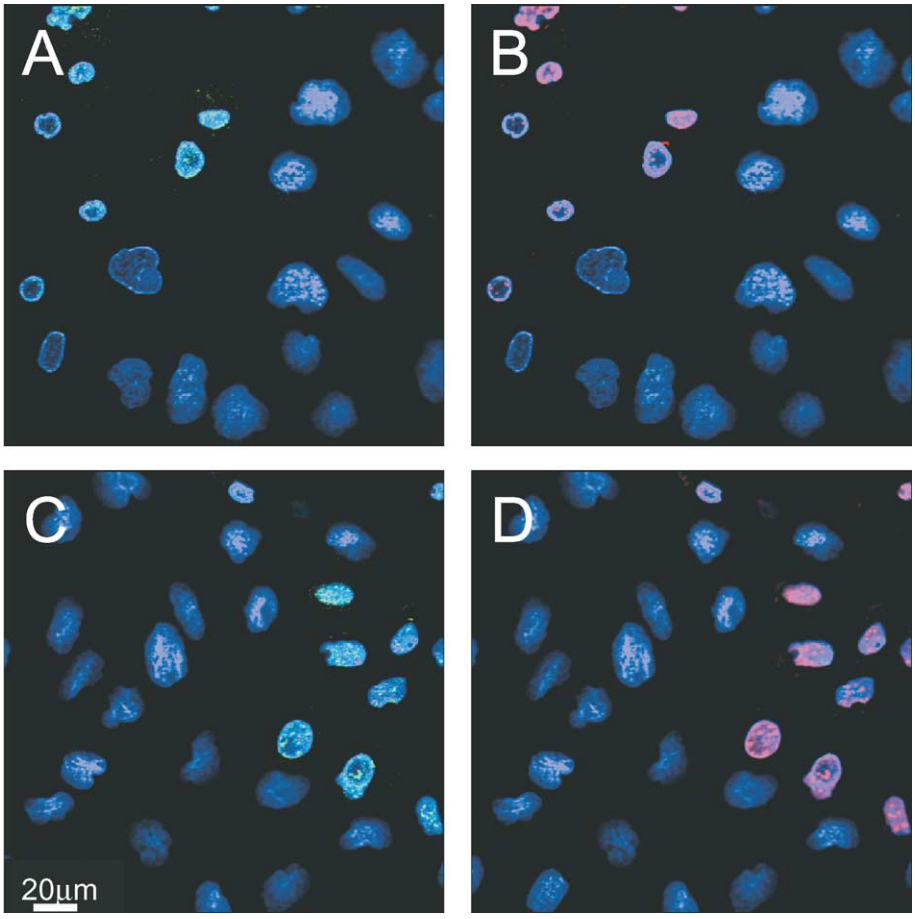

Fig. 5. Plasma membrane integrity of MDCK cells after TAT(44–57)-FM incubation. Cells were grown for 1–2 days on glass cover slips and incubated with 1 μM Cys-TAT(44–57)-FM (10 μM total peptide) and Merck 33342 in medium without FCS for 2 h at 37 °C. Cells were washed and incubated with EthD-1 for further 20 min at 37 °C either immediately (A,B) or after 20 min recovery at 37 °C in medium without FCS (C,D). After washing, cells were mounted without fixation and x, y optical sections were taken through the center of the nuclei. The micrographs focus on areas with peptide uptake. In all cultures, green/red nuclei accounted for $<5\%$ of total nuclei.

peptide). The preparation was immediately scanned after mounting in incubation medium. Neither EthD-1 nor peptide-FM are found in the nuclei or the cytoplasm of these cells. An optical section through the lower part of the cell layer (Fig. 3B) reveals an accumulation of Cys-TAT(44–57)-FM. This localization of peptide close to the cover slip is well recognized in the *x*, *z* and *y*, *z* optical sections in Fig. 3A′, A″, B′ and B″. Findings were similar with 0.2 μM Cys-TAT(44–57)-FM (2 μM total peptide) and with cultures that were washed before mounting in EBSS or medium (not shown).

About 15 min after mounting at room temperature, EthD-1 started to enter part of the cells at both peptide concentrations tested, indicating that the integrity of the plasma membranes became compromised. At the same time, Cys-TAT(44–57)-FM accumulated in the nuclei of these cells. Intact cells were still free of Cys-TAT(44–57)-FM. Fig. 3C–F illustrates foci of compromised cells with EthD-1 and peptide-FM staining.

In preparations with Cys-TAT(37–53)-FM, >50% of the cells were permeable to EthD-1 immediately after mounting (Fig. 4). Peptide-FM was found in the nuclei as well as in the cytoplasm of these cells. Control cultures incubated with 1 μM FM, EthD-1 and Hoechst 33342 contained less than 5% EthD-1 stained nuclei. No FM was detectable within these cells or at the cell borders. Fig. 4E/F shows optical sections through the center of the nuclei focusing on an area containing EthD-1 stained nuclei.

To test whether the observed plasma membrane permeability to Cys-TAT(44–57)-FM and EthD-1 under the above conditions was induced by the peptide or due to nonideal conditions after mounting, cells were incubated for 2 h with 1 μM Cys-TAT(44–57)-FM (10 μM total peptide). All incubations and washing steps were carried out in medium without FCS at 37 °C. After peptide incubation, cultures were washed and incubated with EthD-1 for 20 min. After further washing, cells were mounted and optical sections through the center of the nuclei were immediately taken. No substantial peptide uptake was observed under these conditions. Less than 5% of total nuclei were stained with Cys-TAT(44–57)-FM. The same nuclei were positive for EthD-1 uptake. Fig. 5A,B illustrates a cell culture area containing stained cells. The result was the same after incubation with peptide for 4 h (not shown). The cultures contained a similar percentage of EthD-1 stained cells as control cultures treated with 1 μM FM and EthD-1 (see Fig. 4F). To confirm that cells displaying TAT uptake were irreversibly permeable to EthD-1, cultures were incubated for 20 min in fresh medium at 37 °C after incubation with peptide and before EthD-1 was added. Findings were the same as without the recovery phase (Fig. 5C,D).

4. Discussion

Our findings put the perspectives for TAT peptides as carriers for intracellular targeting into a new light. The TAT peptide conjugates were neither able to cross pure lipid bilayers nor did they enter MDCK cells with integer plasma membranes.

Using a novel assay that allows to visualize entry of aromatic carboxylic acids into liposomes, we could clearly demonstrate that Cys-TAT(44–57) is not able to carry the aromatic carboxylic acid SAM into Tb^{3+}-containing liposomes, independently of the presence or absence of negatively charged lipids in the membrane. This contradicts the hypothesis that TAT peptides permeate lipid membranes without the need of any other mechanisms such as ATPases, carriers, receptors or endocytosis.

The liposomal assay presented in this study is broadly applicable. The only prerequisite is that the studied molecule either contains an aromatic carboxylic acid or that such a moiety can be conjugated to it without changing the membrane permeation properties of the molecule.

Tb^{3+} is an ideal intraliposomal partner for the generation of fluorescence. Tb^{3+}-loaded liposomes were used together with liposomes containing dipicolinic acid in liposome fusion assays [21]. Due to its hydrophilicity and high charge density, Tb^{3+} is localized in the liposomal lumen and neither accumulates in the lipid bilayer nor leaks out of the liposomes. Therefore, fluorescence only occurs upon liposome entry of the molecule under study and not on accumulation in or binding to the membrane.

Complementary studies with living cells in the CLSM showed that no plasma membrane permeation occurred with intact MDCK cells. Cys-TAT(44–57)-FM was not observed in the nuclei or the cytoplasm of living cells after up to 4 h of peptide incubation in medium at 37 °C. Only cells permeable for EthD-1, a marker for plasma membrane impairment, showed uptake and accumulation of Cys-TAT(44–57)-FM in the nuclei. Cells that had taken up the peptide-FM remained permeable to EthD-1 also after 20 min of recovery at 37 °C. In medium at 37 °C, the number of compromised cells was similar to the number of EthD-1 stained cells in a control culture incubated with FM and EthD-1. However, in EBSS at room temperature (conditions after mounting), both the peptide-FM and EthD-1 started to enter a higher number of cells. From this, we conclude that only deteriorating cells are susceptible to TAT(44–57) peptide entry. In contrast to TAT(44–57), TAT(37–53) had toxic effects on the MDCK cells already at a concentration of 2 μM. Also, Vivès et al. [13] found higher toxicity for this peptide as compared to TAT(44–57) in cultures of HeLa cells using an MTT test.

Our observations that TAT(44–57)-FM is not capable to permeate intact plasma membranes of MDCK cells are in line with findings from Violini et al. [22]. They fully agree with our own results from the liposomal approach, the Tb^{3+} assay. There is no evidence that TAT peptides could traverse integer biological membranes by an

unspecific energy-independent mechanism as previously postulated. The discrepancies between these findings and reports on cellular uptake of TAT peptides using other cell lines [13–15] would rather favor the hypothesis of a more specific cell line-dependent mechanism.

It became obvious from our own studies and from investigations by Lundberg and Johansson [23] that microscopic investigations on cellular uptake of CPPs are prone to artifacts, be it from fixation, which is known to produce leakiness, or be it due to the deterioration of unfixed cells in mounted preparations. Furthermore, care has to be taken to differentiate between uptake of peptides into the cells and accumulation at the cell surface. The latter could eventually act as a deposit that enters deteriorating cells after mounting. Using different incubation conditions and by testing the integrity of the cells with EthD-1, we could clearly distinguish between living and deteriorating cells. Analysis of the CLSM data for co-localization of peptide-FM with DNA, i.e. Merck 33342 staining, and the generation of optical *z* sections allowed us to discriminate between nuclear peptide accumulation and peptide deposition at the interface between cells and cover slip.

Unambiguous cell entry has been shown with larger constructs, e.g. fluorescently labeled TAT–protein conjugates. In this case, a vesicular staining of cells is found [14,24], indicating that larger constructs possibly enter by the endocytotic pathway.

The novel liposome assay, together with CLSM studies, will now allow us to check further CPPs for their ability to cross lipid bilayers and to enter intact cells.

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