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Research Article

Enhanced heparan sulfate proteoglycan-mediated uptake of cell-penetrating peptide-modified liposomes

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Abstract. Protein transduction domains (PTDs) are used to enhance cellular uptake of drugs, proteins, polynucleotides or liposomes. In this study, functionalized Antennapedia (Antp, aa 43–58) and HIV Tat (aa 47–57) peptides were coupled to small unilamellar liposomes via thiol-maleimide linkage. Modified liposomes showed higher uptake into a panel of cell lines including tumor and dendritic cells than unmodified control liposomes. Liposome uptake was time and concentration dependent as analyzed by flow cytometry and live-cell microscopy. At least 100 PTD molecules per small unilamellar liposome (100 ± 30 nm) were necessary for efficient translocation into cells. Cellular uptake of PTD-modified liposomes was 15- to 25-fold increased compared to unmodified liposomes and was inhibited by preincubation of liposomes with heparin. Glycosaminoglycan-deficient CHO cells showed dramatically reduced cell association of PTD-modified liposomes, confirming the important role of heparan sulfate proteoglycans in PTD-mediated uptake. Antp-liposomes used as carriers of the cytotoxic drug N^4-octadecyl-1-β-D-arabinofuranosylcytosine-(5′-5′)-3′-C-ethinylcytidine showed a reduction of the IC_{50} by 70% on B16F1 melanoma cells compared with unmodified liposomes. PTD-functionalized liposomes, particularly Antp-liposomes, represent an interesting novel carrier system for enhanced cell-specific delivery of a large variety of liposome-entrapped molecules.

Key words. Antennapedia; HIV-Tat; peptide-liposome; heparan sulfate proteoglycan; cytotoxic liposome.

Uptake of pharmaceutically active molecules into living cells is hampered by the lipophilic nature of the plasma membrane. Large molecules such as proteins, peptides and oligonucleotides are generally poorly taken up by cells since they do not cross the lipid bilayer of the plasma membrane efficiently. Strategies to improve membrane permeability play an important role in the development of new drug delivery systems. Specific domains of several proteins, so-called protein transduction domains (PTDs), efficiently pass through biological membranes [1]. PTDs were first identified while investigating the spontaneous cell entry of the HIV Tat protein and its subsequent translocation to the nucleus. A short sequence rich in basic amino acids is responsible for cellular uptake of this protein [2, 3]. Similar properties were found for Antennapedia (Antp), a *Drosophila* homeodomain transcription factor [4] and in the Herpes virus protein VP22 [5]. The peptide sequence Antp (43–58), corresponding to the third helix of the homeodomain of Antennapedia, has been shown to promote translocation across cellular membranes. Due to their basic sequence, these and related basic peptides are rapidly internalized by mammalian cells and therefore used as delivery vectors [6].
Colloidal drug carriers such as liposomes and target-specific immunoliposomes are widely used as delivery sys-

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tems for a broad spectrum of agents including chemotherapeutics, imaging agents, antigens, lipids and DNA [7–10]. These delivery systems have several therapeutic advantages, such as the ability to transport large drug concentrations to a specific site, sparing healthy tissue from toxic effects and increasing the systemic drug circulation time. Therapeutic molecules are protected by the lipid bilayer of the liposome from metabolism and enzymatic degradation, a feature that is particularly important for in vivo applications. To improve cellular uptake of liposomes, we developed a simple and versatile method to link C-terminally functionalized Antp and Tat peptides via a covalent cysteine-maleimide coupling reaction to the outer surface of liposomes [11].

There is still controversy about the mechanism responsible for cell membrane translocation of PTDs and it may vary among the various PTDs and depend on the structure and composition of the target cell membranes. Recent publications have proposed that PTD-mediated membrane translocation is an energy-dependent process that may be a direct consequence of peptide association with heparan sulfate proteoglycans (HSPGs) [12]. Drin et al. [13] showed that internalization of SynB5, a peptide derived from protegrin and the Antp peptide is a temperature- and energy-dependent process with endosomal transport as a key component of the mechanism. We [14] and others [15] have demonstrated that PTDs facilitate cell uptake of cargo molecules via endocytosis and require the expression of negatively charged glycosaminoglycans on the cell surface. Another detailed study demonstrated that the Tat peptide interferes with the release of newly synthesized HPSGs from cells [16]. One elegant study showed recently that the most likely route for PTD-mediated cellular uptake is via lipid raft-mediated macropinocytosis [17]. Most studies involving PTD-mediated cellular uptake have been carried out with Tat peptide. Tat-modified nanoparticles, such as cross-linked iron oxide for cell tracking by magnetic resonance [18] or polymeric drug carriers [19] showed improved uptake properties when compared to unmodified particles. The Tat peptide was also used to investigate PTD-mediated liposome uptake. As shown by Torchilin et al. [20], conventional and polyethyleneglycol-modified Tat-liposomes were taken up by different cell types, provided that 100–500 peptide molecules were attached per liposome. In a subsequent paper, the same authors described the cell transfection properties of Tat-liposome/DNA complexes and reported high rates of green fluorescent protein expression at lower cytotoxicity compared with common commercial transfection reagents [21]. The Antp peptide was also used to deliver a poorly antigenic MHC class I-restricted peptide to antigen-presenting cells to elicit a cytotoxic T lymphocyte immune response. A recombinant Antp antigenic fusion peptide was encapsulated in liposomes to prevent peptide degradation, enhance cytosolic delivery and antigen presentation on dendritic cells [22, 23].

The work we present here illustrates the properties of Antp- and Tat-modified small unilamellar liposomes (Antp-L, Tat-L) and their ability to bind to and translocate into different cell types, such as CHO, B16 melanoma, F9 teratocarcinoma and dendritic cells. We show that at least 110–136 peptide molecules per liposome are necessary for effective cell translocation and that cell uptake of the PTD-modified liposomes is strongly dependent on glycosaminoglycan expression on the target cells, confirming the important role of HSPGs in PTD-dependent cellular uptake. Thus, PTD-modified liposomes, and particularly Antp-L, provide a novel system for enhanced delivery of a large variety of liposome-entrapped molecules into cells.

Materials and methods

Chemicals

Soy phosphatidylcholine (SPC) was obtained from L. Meyer (Hamburg, Germany). Cholesterol (CHOL) was purchased from Fluka (Buchs, Switzerland). 2-Dipalmitoyl-sn-glycerol-3-phosphatidylethanolamine (DPPE) and methoxy-polyethyleneglycol-phosphatidylethanolamine ($PE-PEG_{2000}-OMet$) were from Sygena (Liestal, Switzerland) and amino-polyethyleneglycol-phosphatidyl-ethanolamine ($PE-PEG_{2000}-NH_2$) from Shearwater Polymers (Enschede, The Netherlands). The bifunctional coupling reagent sulfosuccinimidyl 4-[N-maleimidoethyl]-cyclohexane-1-carboxylate (sulfo-SMCC) was from Pierce (Lausanne, Switzerland). The fluorescent dye 3,3′-dioctadecyl-oxacarbocyanine perchlorate (DiO) and Alexa Fluor 546 transferrin were from Molecular Probes (Eugene, Ore.). WST-1 reagent was from Roche Diagnostics (Mannheim, Germany). The new cytotoxic heterodinucleoside dimer N^4-octadecyl-1-β-D-arabinofuranosylcytosine-(5′-5′)-3′-C-ethinylcytidine (NOAC-ETC) was synthesized as described for similar dimers [24]. C-terminal cysteine-modified Antp- and TAT-peptides and correspondingly FITC-labeled Antp and TAT were synthesized by Neosystems (Strasbourg, France). The C-terminal cysteine-modified Tat peptide was also synthesized as described by Console et al. [14]. Peptides without FITC contained N-terminal biotin. Heparin (a heparan sulfate fraction) was a kind gift from H. P. Wessels (Hoffmann-La Roche, Basel, Switzerland). Dulbecco's modified Eagle's medium (DMEM) was from Sigma (Buchs, Switzerland). Opti-MEM-1, fetal bovine serum (FBS) and all culture media supplements were from Invitrogen (Basel, Switzerland). Ham's F12 medium was from Bioconcept (Allschwil, Switzerland). All buffer salts and other chemicals were of analytical grade and obtained from Fluka, Sigma (Buchs, Switzerland) or Merck (Darmstadt, Germany).

Cells

Murine B16F1 melanoma, murine F9 teratocarcinoma, human HeLa and W-38 fibroblast cells were maintained in DMEM containing 10% FBS, 1% L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 U/ml nystatin. Wild-type Chinese hamster ovary cells CHO K1, and the mutants CHO pgs A-677, and CHO pgs A-745 were from ATCC (Manassas, Va.) and maintained in Ham's F12 medium. Mouse dendritic cells (DCs) were a kind gift from B. Ludewig (Kantonsspital, St. Gallen, Switzerland) and purified as described elsewhere [25].

Liposome preparation, modification and labeling

The basic composition of the liposomes was SPC:CHOL:D,L α-tocopherol at a molar ratio of 1:0.2:0.001. All additional compounds were added in mol parts refering to SPC as the main lipid. For maleimide modification, DPPE was added at 0.035 mol parts. For fluorescence-labeled liposomes, the lipophilic dye DiO (0.004 mol parts) was included, whereas for the cytotoxicity studies, the duplex drug NOAC-ETC (0.058 mol parts) was added to the lipid mixture. PEG-liposomes were prepared by addition of 0.035 mol parts PE-PEG_{2000}-OMet and 0.035 mol parts PE-PEG_{2000}-NH_2 to the basic lipid mixture. Small unilamellar liposomes (SUVs) were prepared by repeated sequential filter extrusion of multilamellar liposomal preparations in phosphate buffer (PB, 67 mM, pH 7.4) through Nuclepore membranes (Sterico, Dietikon, Switzerland) of 0.2- and 0.1-µm pore diameter with a Lipex extruder (Lipex Biomembranes, Vancouver, Canada) [11]. Size and stability of the liposomes were analyzed with a Nicomp particle sizer (Model 370; Santa Barbara, Calif.).

Preparation of peptide-modified liposomes

Liposomes containing 0.035 mol parts DPPE or PE-PEG-NH_2 in PB were incubated with crystalline sulfo-SMCC at a molar ratio of liposomal amino to succinimide groups of 1:5 for 30 min at 30 °C. Excess sulfo-SMCC was removed by overnight dialysis at 4 °C against PB. Peptides (Antp or Tat) were incubated under stirring with 1 ml maleimide-modified liposomes (80 mg/ml SPC) in PB buffer for 48 h at 25 °C. Control liposomes (cys-L) were modified with a tenfold molar excess of cysteine to block all maleimide groups. To prevent dimerization of the peptides, the coupling reaction was made in the presence of the reducing agent tributylphosphine (2 mM) and kept under a nitrogen atmosphere. Non-reacted peptides were removed by extensive dialysis at 4 °C against PB.

Determination of the number of peptide molecules attached to liposomes

FITC trace-labeled peptides were incubated with maleimide-modified liposomes at molar ratios of maleimide to peptide-thiol groups of 1:0.12, 1:0.24, 1:0.60 and 1:1.20 as described above. The coupling efficiency was measured in 96-well plates using a Tecan multiplate reader fluorometer (Tecan Ultra Evolution; Tecan, Männedorf, Switzerland) using 485-nm excitation and 535-nm emission filters. The numbers of coupled peptide molecules per liposome were determined by applying the calculation method as described by Marty and Schwendener [26] for liposomes of a mean diameter of 100 nm. Briefly, model calculations of numbers of liposomes and reactive groups are based on experimentally determined mean hydrodynamic diameters of the liposomes and from assumptions on spherical vesicle geometry parameters. Using these parameters, one can approximate liposome numbers per volume and, e.g. the numbers of amino groups available for peptide modification on one liposome of a given composition and mean diameter.

Flow cytometry

All cells (10^5 cells per well in 12-well plates) were cultured in growth medium for 24 h at 37 °C and 5% CO_2. The medium was removed and cells were incubated in serum-free OptiMEM-1 medium for 30 min at 37 °C. DiO-labeled liposomes (Antp-L, Tat-L, cys-L; 500 nmol total lipid/ml each) were incubated at 37 °C and 5% CO_2 for 90 min with cells at different peptide-coupling ratios (1:0.24; 1:0.6 and 1:1.2) or with liposomes containing the same total number of peptide molecules attached at different molar maleimide to peptide ratios. Cells were also incubated with dilutions of peptide liposomes prepared at the 1:1.2 ratio (15–500 nmol lipid/ml medium) or incubated for different time periods (5 min–24 h). Liposome association to different cell lines was assessed by incubation of the cells with liposomes at 500 nmol lipid/ml medium for 90 min. Heparin-mediated inhibition of liposome association was investigated by preincubation of the liposomes with 20 µg/ml heparin for 15 min before incubation with cells. Liposomes were removed and cells were kept in growth medium for 1 h at 37 °C and 5% CO_2. Then, the cells were washed with PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), detached with 10 mM EDTA in PBS and fixed in 3.7% formaldehyde in PBS. In some additional experiments, cells were treated with trypsin (0.5 mg/ml) before flow cytometric analysis. This treatment reduced the fluorescence intensity of cell-associated liposomes up to 70%, presumably due to loss of cell surface-bound liposomes. Cell-associated fluorescence of the liposomes was measured using a FACScan (Becton Dickinson, Basel, Switzerland) using the CELLQuest software. Experiments were carried out in triplicate by analysis of 10,000 cells per sample.

Live cell fluorescence microscopy

HeLa cells were grown on glass coverslips to 50–80% confluency. DiO-labeled liposomes and Alexa Fluor 546

transferrin added as a marker for receptor-mediated endocytosis were incubated with cells in OptiMEM-1 for 1 h at 37 °C in a CO_2 incubator. Cellular uptake was monitored in live cells on an Olympus Biosystems widefield fluorescence microscope (OBS, Munich, Germany). Twenty-four images in the z-axis were acquired and deconvoluted with the Autodeblur software (Autoquant, Watervliet, N. Y.). Imaris image-processing software (Bitplane, Zürich, Switzerland) was used for further image analysis.

Cytotoxicity tests

B16F1 cells (10,000 cells per well in 96-well plates) were seeded in growth medium for 24 h and cultured at 37 °C and 5% CO_2. Liposomes containing NOAC-ETC at concentrations of 1.6 – 100 µM in PB were diluted in DMEM medium 1:1 (v/v) and added to the cells for 30 min, 2 and 4 h. Liposomes and heparin (20 µg/ml) were preincubated for 15 min before being added to the cells in the corresponding assay. After the indicated incubation times, the liposome-containing medium was removed and replaced with growth medium for 24 h. Cell viability was determined with the WST-1 test using a Dynatech MR 4000 plate reader. All measurements were carried out in quadruplicate. The 50% growth-inhibitory concentration IC_{50} was calculated from interpolations of the graphical data.

Results

Preparation and characterization of peptide-modified liposomes

We studied cellular uptake of liposomes modified with the two highly basic peptide sequences derived from Antp (SGRQIKIWFQNRRMKWKKC) and HIV Tat (SGYGRKKRRQRRRC), respectively. The peptides were chemically synthesized, biotinylated at the amino terminus and functionalized at the carboxy terminus with a cysteine residue to allow covalent coupling to liposomes. Small unilamellar liposomes were of 100 ± 30 nm mean diameter and produced by a filter extrusion technique described before [11]. In a first set of experiments, we analyzed whether the total number of peptide molecules attached per liposome influences the binding and uptake properties of the liposomes. Liposomes containing various numbers of peptide molecules linked to the surface were prepared. The coupling efficiency was determined with FITC-labeled peptide included as trace label. As summarized in table 1, coupling reactions performed at molar ratios of peptide to maleimide groups on liposomes of 0.12, 0.24, 0.6, and 1.2 resulted in an average of 0, 36, 69 and 110 Antp peptide molecules and 0, 45, 60 and 136 Tat peptide molecules, respectively, attached per liposome.

Table 1. Determination of peptide numbers attached to liposomes.

Maleimide:peptide (mol ratio)	1:0.12	1:0.24	1:0.6	1:1.2
Antp peptide per liposome	n.d.	36 ± 9	69 ± 16	110 ± 14
Tat peptide per liposome	n.d.	45 ± 1	60 ± 4	136 ± 5

Sulfo-SMCC-modified liposomes were incubated with cysteine-modified peptides containing trace labeled FITC-peptide as described in Materials and methods. After removing non-reacted peptides by dialysis, the number of peptide molecules attached to the surface of a liposome was determined by fluorescence spectroscopy and application of the calculation parameters as described by Marty and Schwendener [26]. n.d., not detectable.

Next, we investigated cell binding of Antp- and Tat-modified liposomes carrying different numbers of coupled peptides by flow cytometry. Flow-cytometric analysis cannot distinguish between cell surface-associated and internalized liposomes and allows only the determination of average fluorescence values in a large cell population. Thus, we use the term 'association' for the description of the liposome cell interaction measured by flow cytometry. Increasing numbers of peptide molecules linked to liposomes (table 1) effectively enhanced association to B16F1 cells (fig. 1 A). Liposomes modified with an average of 110 Antp molecules, corresponding to the 1 to 1.2 molar coupling reaction ratio of maleimide to peptide, resulted in the highest association to B16F1 cells. Cell binding of liposomes modified with approximately 136 copies of Tat peptide was reduced by two-thirds relative to Antp-L. Lower peptide concentrations used for the coupling reaction, i. e. liposomes modified with less than 100 peptides, were taken up at considerably lower efficiency (fig. 1 A).

To further analyze the binding properties of the two types of liposomes, cells were incubated with two and five times the amount of liposomes that were modified with 0.6 and 0.24 moles of peptide, corresponding to 60 – 70 and 35 – 45 peptide molecules per liposome, respectively. As shown in figure 1 B, increasing the liposome number did not result in equivalent cell association. Thus, for all further experiments, the liposomes were modified with the highest molar liposome to peptide ratio of 1 to 1.2 resulting in 110 ± 14 Antp and 136 ± 5 Tat peptides per liposome. Time- and concentration-dependent association to B16F1 cells are shown in figure 2. Association of Antp-L with B16F1 cells was considerably more efficient than with Tat-L or cys-L (fig. 2). Taken together, our data show that a minimal number of peptide molecules per liposome is required for efficient cellular uptake. The addition of more liposomes modified with fewer peptide molecules did not result in comparable cell association (fig. 1 B). Similar results were obtained with F9 and W38 cells (data not shown). Fixation of cells did not influence the fluorescence signal in flow cytometry (data not shown).

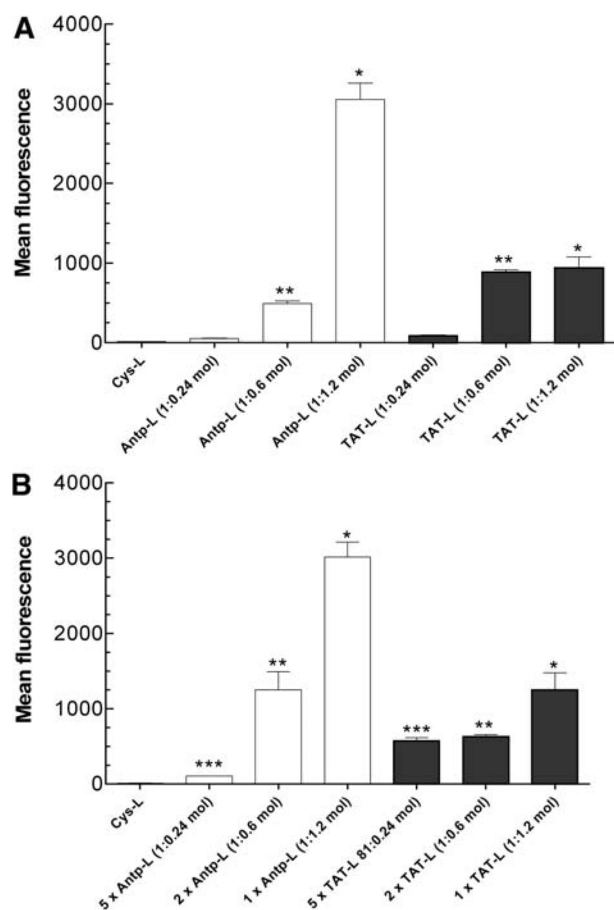

Figure 1. Dependence of liposome association with B16F1 cells on peptide derivatization. *(A)* Association of liposomes modified with different numbers of peptide molecules depending on the incubation ratio of maleimide groups to peptide (1:1.2, 1:0.6, 1:0.24 and 1:0.12 mol) at a concentration of 500 nmol total lipid/ml medium. *, ** p = 0.001. *(B)* Association of Antp-L and Tat-L modified with different numbers of peptides and incubated at a constant peptide to cell ratio. * p = 0.0005; * p = 0.0111; *** p < 0.0001. The data are expressed as mean ± SD (n = 3), Cys-L, cys-liposomes; Antp-L, Antp-liposomes; Tat-L, Tat-liposomes.

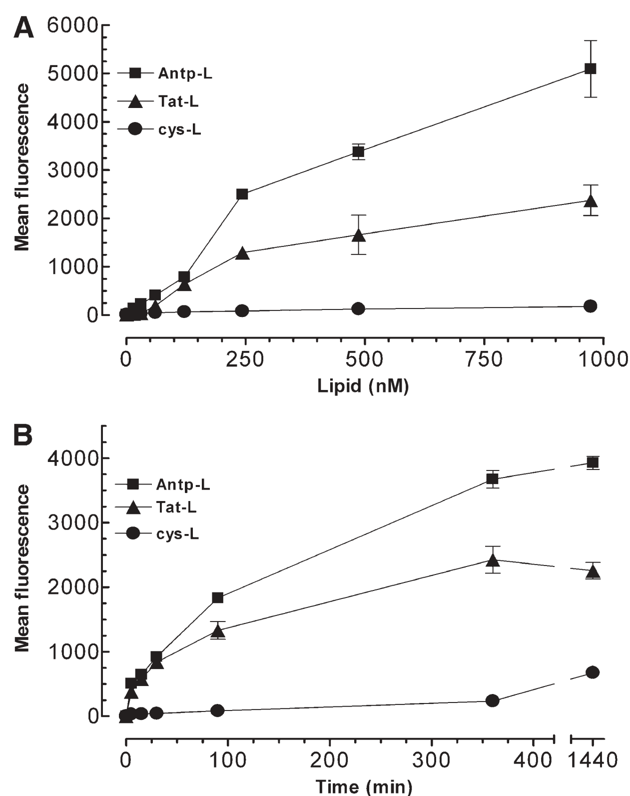

Figure 2. Concentration (*A*) and time (*B*)-dependent association of different liposome formulations with B16F1 cells. Cells (10^5 per well) were incubated with fluorescence-labeled Antp-L (squares), Tat-L (triangles) or cys-L (circles) at a final concentration of 15–900 nmol total lipid /ml for 90 min (*A*) or at 250 nmol lipid/ml for 5 min to 24 h (*B*). Data are expressed as the mean ± SD (n = 3).

Microscopic analysis of liposome uptake

Cellular uptake of PTD-modified liposomes was further investigated microscopically in live cells. As shown in figure 3 A, cys-L were internalized into HeLa cells and accumulated in intracellular vesicular structures, presumably early and late endosomes that were enriched in transferrin added as a marker for endocytosis. Due to the fact that cell association of cys-L was less efficient than PTD-liposomes (cf. fig. 2), the exposure time in figure 3 A was approximately ten times longer to visualize equally the cell distribution of cys-L and peptide-modified liposomes. Antp- and Tat-L accumulated to a lower extent in endosomes and remained associated with the cell surface and with intracellular vesicular structures devoid of transferrin (fig. 3 B, C). At late time points, Tat-L accumulated also in large aggregates on the cell surface while Antp-L were enriched in small intracellular vesicular structures (data not shown). This suggests alternative routes of cell entry for cys- and PTD-derivatized liposomes.

Mechanism of cellular uptake

We next compared the association properties of Antp-L and Tat-L in various cell lines. As summarized in figure 4, the fluorescence intensity of Antp-L was highest in B16F1 melanoma cells, followed by DC and W38 fibroblasts. In contrast, Tat-L fluorescence was reduced by about 60% in B16F1, 30% in DCs and 24% in W38 cells, whereas in CHOK1 cells, Tat-mediated liposome association was similar to the level determined for Antp-L in B16F1 cells. This finding suggests that cell surface expression of HSPGs differs between cell lines either in the number of molecules per cell or in the structure of the HSPGs exposed on the cell surface.

To investigate further the putative mechanism responsible for PTD-mediated cellular uptake, we preincubated two types of Antp-L, conventional liposomes and PEG-liposomes, with 20 µg/ml heparin prior to addition to B16F1 cells. As shown in figure 5 heparin inhibited cellular up-

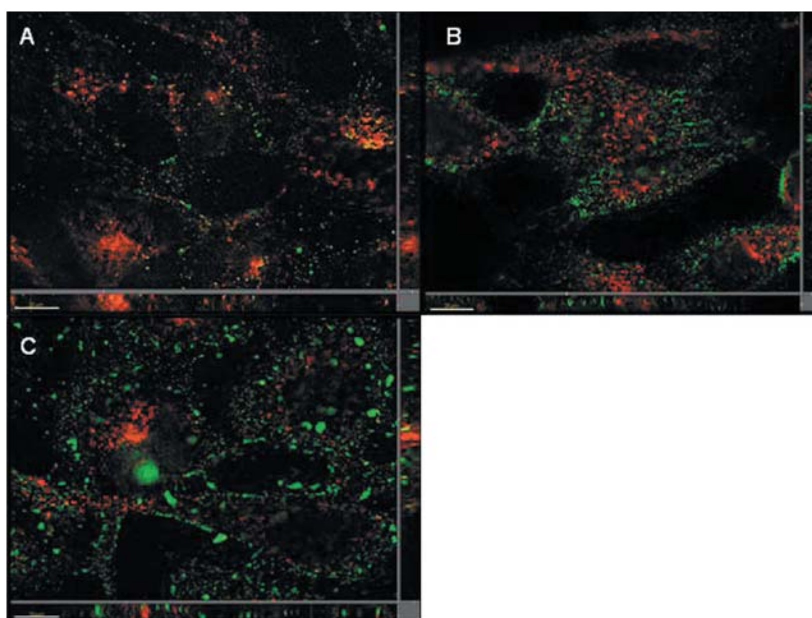

Figure 3. Cell association and internalization of fluorescence-labeled liposomes studied in live HeLa cells. Cells were incubated at 37 °C with DiO-labeled cys-L (*A*), Antp-L (*B*) and Tat-L (*C*) for 1 h. Alexa 546 transferrin (25 µg/ml) was added as a marker for endocytosis. DiO and transferrin fluorescence are shown in green and red, respectively. The square panel in each figure represents a representative x/y plane, the rectangular pictures at the bottom and on the right of each figure show x/z and y/z sections, respectively. The exposure time of *A* was approx. ten times longer than of *B* and *C*. Bar, 20 µm.

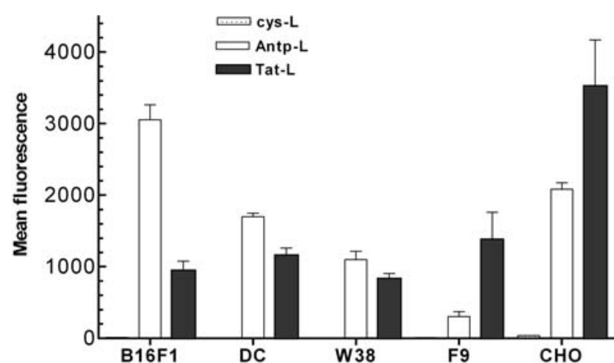

Figure 4. Liposome association with different cell types. Cells were incubated with fluorescence-labeled cys-L, Antp-L or Tat-L at concentrations of 500 nmol total lipid/ml medium for 90 min at 37 °C. Cell association of liposomes was analyzed by flow cytometry. Data are expressed as the mean ± SD (n = 3). Due to the low fluorescence, the bars of cys-L are not visible in the B16F1, DC, W38 and F9 cells.

take of both types of Antp-modified liposomes. PEG-modified liposomes bound less efficiently to cells, presumably due to the interference of PEG with binding to the HSPGs. Comparable results were obtained with Tat-L (data not shown). Cell association of liposomes was also inhibited by preincubation of liposomes with dextran sulfate and chondroitin sulfate A as shown before [14].

The involvement of HSPG in binding of liposomes to cells was further demonstrated with two mutant CHO cell lines. CHO pgs A-745 cells lack UDP-D-xylose:serine beta-1,3-D-xylosyltransferase and are therefore deficient in the synthesis of all proteoglycans. CHO pgs D-677 cells express a reduced amount of HSPG due to a tenfold reduction of N-acetyl-glucosaminyl- and glucuronosyl-transferase activities. As shown in figure 6, reduced uptake of both Antp-L and Tat-L was observed with the mutants CHO pgs 677 and CHO pgs 745 compared with the CHO K1 cells. We conclude that cell surface-expressed HSPGs play an important role in the association and subsequent uptake of PTD-modified liposomes by various cell types, the exact mechanism remaining to be determined.

Cytotoxic activity of peptide-modified liposomes

Due to their rapid cellular uptake (fig. 2), PTD-modified liposomes might be a useful tool to deliver drugs into cells. To demonstrate the advantage of increased cellular uptake of a cytotoxic compound, we compared the effect of Antp-L with cys-L loaded with NOAC-ETC on B16F1 cells. NOAC-ETC is a heterodimeric drug composed of the lipophilic derivative of cytosine arabinoside (NOAC) and the highly active antitumor drug ethinylcytidine [27, 28]. By treating B16F1 cells with drug-containing Antp-L, the IC_{50} of NOAC-ETC was reduced after 2 h incubation by 40 % from 38 to 24 µM and after 4 h by 70 % from 17 to 5 µM compared to cys-L. Unexpectedly, the reduction of the IC_{50} observed with Antp-L was lower than an-

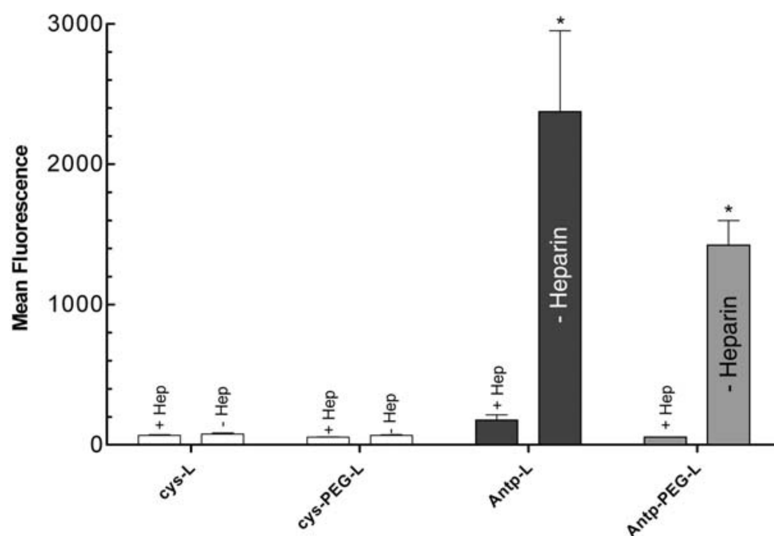

Figure 5. Inhibition of liposome uptake by heparin. Antp-L and Antp-PEG-liposomes (500 nmol total lipid/ml) were preincubated with 20 µg/ml heparin for 15 min before incubation with B16F1 cells for 60 min at 37 °C. Cell association of fluorescence-labeled liposomes was analyzed by flow cytometry. Data are expressed as the mean ± SD (n = 3); * $p = 0.0526$.

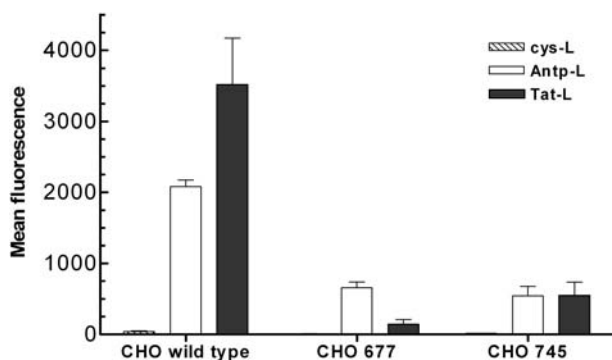

Figure 6. Involvement of HSPGs in the cell association of PTD liposomes. Antp-L (open bars), Tat-L (black bars) and cys-L (hatched bars) at 500 nmol total lipid/ml were incubated for 90 min with CHO-K1 cells and the mutants CHO-677 and CHO-745. Cell association of fluorescence-labeled liposomes was analyzed by flow cytometry. Data are expressed as the mean ± SD (n = 3). Due to the low fluorescence, the bars of cys-L are not visible in the CHO mutant cells.

ticipated from the flow cytometry experiments (see fig. 4), where the difference of cell-associated liposomes between cys-L and Antp-L on B16F10 cells was more pronounced. The reduced cytotoxic effect of Antp-L could be explained by the prodrug nature of NOAC-ETC requiring cleavage of the dimeric molecule for cytotoxic activation. Preincubation of Antp-L or Tat-L loaded with NOAC-ETC with heparin reduced the cytotoxic effect on B16F1 cells as shown in figure 7B. These results further corroborate the importance of HSPGs on cellular uptake of PTD-modified liposomes.

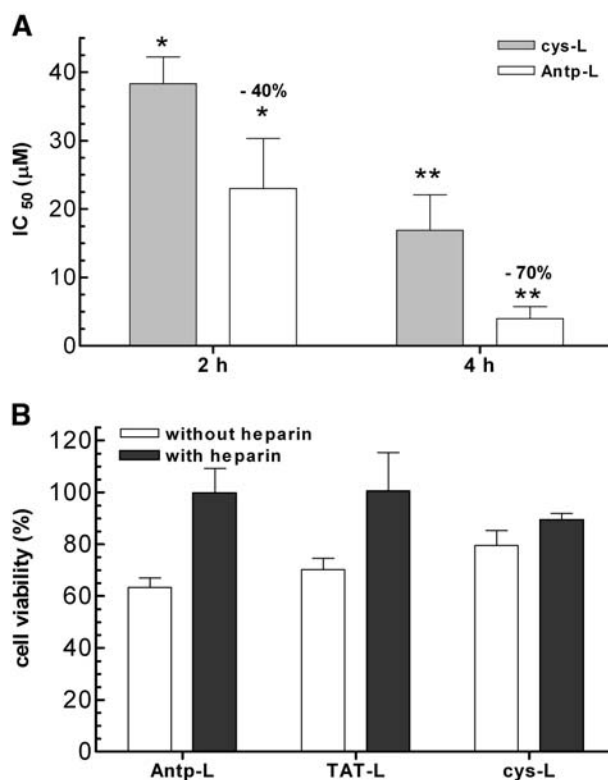

Figure 7. Cytotoxicity of drug-loaded liposomes in B16F1 melanoma cells. (*A*) B16F1 cells were incubated with cys-L and Antp-L loaded with NOAC-ETC for 2 and 4 h. Cell viability was measured using the WST-1 test after 24 h incubation at 37 °C. The IC_{50} values were extrapolated from graphical plots of the viability curves. Data are expressed as the mean ± SD (n = 3); * $p < 0.0001$; ** $p < 0.002$. (*B*) Cys-L, Tat-L and Antp-L loaded with NOAC-ETC were preincubated with heparin (20 µg/ml) for 15 min followed by addition to B16F1 cells for 30 min. Cell viability was measured using the WST-1 test after 24 h incubation at 37 °C and 5 % CO_2.

Discussion

In this study we show that small nanosized cargo particles such as liposomes functionalized with PTD peptides promote efficient delivery of entrapped molecules such as lipophilic fluorescent dyes and cytotoxic drugs into live cells. In this system, the liposome functions as a nanocage providing a protected compartment carrying bioactive molecules, while the peptide bound to the surface of liposomes facilitates cell association and promotes transport across the plasma membrane. We used a simple, versatile and effective site-specific coupling strategy where peptides are modified at their carboxy terminus with cysteine residues. By reaction with maleimide groups introduced on the liposome surface, peptides are attached to the outer liposome bilayer membrane via a stable thioether bond endowing the peptide with a high degree of motional freedom to interact with target molecules expressed on the surface of cells. The number of peptide molecules attached to a small liposome of 100 nm average diameter seems to be very important. Whereas an average of 110–136 peptide molecules linked per liposome promoted strong cell association and internalization, liposomes decorated with less than 70 peptides (fig. 1) were not taken up efficiently.

Since there are no reports in the literature that Antp peptides have been linked to liposomes, our findings can only be evaluated against Tat-modified liposomes as reported by Torchilin et al. [20, 21]. These authors developed Tat peptide-liposome-DNA complexes as transfection vehicles for gene therapy. In vitro transfection of different cell types with these vehicles was effective and less toxic when compared with commercially available transfection reagents. Because Tat peptides were attached to the liposome surface by a non-specific method where peptide amino groups reacted randomly with a p-nitrophenylcarbonyl-phosphatidyl-ethanolamine lipid, up to 500 copies of Tat peptide per liposome were necessary to obtain effective cell translocation [20]. Thus, we conclude that our site-specific thiol-maleimide coupling method has several advantages over methods where a high fraction of the peptides may be sterically hindered from binding to cellular target proteins due to unordered attachment to the liposome surface.

Our microscope data obtained in live cells show that liposomes are internalized by cells. Live-cell imaging was chosen to exclude possible fixation artifacts. The distribution of liposomes on the cell surface and in intracellular vesicles apparently varies among the different types of liposomes used in this study. Images taken from control cys-L were exposed approximately ten times longer to achieve equal fluorescence intensity, reflecting the fact that the PTDs promoted accelerated binding to and uptake into cells. Cys-L accumulated in large vesicles resembling late endosomes that also contained transferrin, as indicated by the fact that green and red vesicles colocalized, giving rise to orange cellular staining (fig. 3A). PTD-derivatized liposomes, on the other hand, accumulated in small vesicle-like structures with a large proportion of material still associated with the plasma membrane even after prolonged exposure (data not shown). As in the controls, the transferrin marker accumulated in large intracellular vesicles that are visible in the red channel in figure 3B, C. Whether the PTD-liposomes released their content at the plasma membrane without entering the endocytotic pathway as the cys-liposomes is an interesting possibility that is the subject of ongoing work. PTD-liposomes might bypass endocytosis and therefore favor cytoplasmic drug delivery into cells. To define the localization and uptake mechanism of PTD and control liposomes, a detailed analysis of liposome trafficking in live cells is underway. This might open new opportunities for liposome targeting to specific cellular compartments. The mechanism responsible for PTD translocation across membranes is still the subject of controversial discussions and may vary among the various PTDs. In earlier publications, the mechanism was proposed as energy independent and preferentially mediated via direct interaction of PTDs with the lipid bilayer of cell membranes [13, 29]. In a recent study, Drin et al. [30] and Letoha et al. [31] proposed that for Antp (aa 43–58) and derivatives thereof cell binding is not only promoted by HSPGs. Such peptides also directly bind to lipids and may thereby cause membrane association due to their amphipathic properties. However, there is increasing evidence that HSPGs play a paramount role as the cellular entry gateway of positively charged molecules and synthetic delivery vehicles. HSPGs function as a natural entry mechanism for polyamines [32], viruses [33], polybasic peptides and polycation nucleic acid complexes [4] and, as shown here, for liposomes carrying positively charged peptides. Independently of the mechanism of internalization, the potential of PTD-conjugated carrier systems to deliver pharmacologically active molecules into almost any cell type in vitro and in vivo suggests the existence of a ubiquitous transport pathway that can be exploited for cargo delivery [34].

We reported earlier the influence of HSPGs on cellular uptake of Tat and Antp peptides and peptide-avidin complexes [14] and our data were recently confirmed by a report showing that entry is mediated by lipid rafts in a process called macropinocytosis [17]. Here, we demonstrate that the uptake of peptide-modified liposomes is also inhibited upon blocking of cellular HSPGs with soluble heparin. In addition, uptake of Antp-L and Tat-L is dramatically reduced in mutant cells defective in glycosaminoglycan synthesis. Interestingly, we found a distinct pattern of cell association between Antp-L and Tat-L on different cell types. These peptides conceivably follow different binding and uptake mechanisms depending on

the variability of the HSPG expression pattern in various cell types. Furthermore, the distinct physicochemical properties of Antp and Tat, such as hydrophobicity and amphipathicity of Antp in contrast to the high polarity of Tat, may also contribute to the characteristic pattern of uptake and translocation observed in different cells (fig. 4). A particularly interesting application of Antp-L and Tat-L could be their utilization as carriers for antigens in vaccine development. Vaccines are predominantly applied via subcutaneous or intradermal injection routes. We demonstrated the feasibility of conventional, unmodified antigen-carrying liposomes as vaccines in the lymphocytic choriomeningitis virus model [25]. After cutaneous application of antigen-carrying Antp-L, such vesicles may be taken up at enhanced rates by antigen-presenting dendritic cells located in the dermal tissue. For parenteral application, liposomes are routinely prepared with PEG-modified lipids to prolong their blood circulation time. We modified PEG-liposomes by attaching Antp peptide molecules at the end of the PEG chain. Such Antp-PEG-liposomes were still able to associate with cell membranes, however, with reduced efficiency, as shown in figure 5. For in vivo use of such cargo vehicles, the covalent attachment of antibodies or antibody fragments to liposomes may be further required for target-specific delivery, because PTD-modified cargo vehicles do not discriminate between the various cell types they encounter after parenteral application. The likelihood that PTD-modified liposomes bind in an unspecific manner to HSPGs expressed on endothelial cells of blood vessels, in the extracellular matrix or on other cell surfaces is high. Therefore, the construction of a drug carrier device combining high target specificity with fast and efficient cell surface association and membrane translocation is an attractive possibility. The liposome scaffold offers a large surface area to accommodate a high number of different functional groups allowing polyvalent coupling reactions. We previously showed improved targeting of scFv antibody-modified liposomes to tumor tissue in animals using the thiol-maleimide coupling strategy [11]. We are therefore pursuing the possibility to simultaneously modify liposomes with PTD peptides and target cell-specific antibodies. Such multifunctional liposomes might allow enhanced delivery of pharmaceuticals for therapeutic and diagnostic intervention into diseased tissue. The exact mechanism of HSPG function in PTD-peptide-modified liposomes or nanoparticle cell binding and translocation remains to be further investigated and offers exciting opportunities for future research in the field of targeted drug delivery.

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