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Transduction of Peptides and Proteins Into Live Cells by Cell Penetrating Peptides

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ABSTRACT

Internalization of peptides and proteins into live cells is an essential prerequisite for studies on intracellular signal pathways, for treatment of certain microbial diseases and for signal transduction therapy, especially for cancer treatment. Cell penetrating peptides (CPPs) facilitate the transport of cargo-proteins through the cell membrane into live cells. CPPs which allow formation of non-covalent complexes with the cargo are used primarily in this study due to the relatively easy handling procedure. Efficiency of the protein uptake is estimated qualitatively by fluorescence microscopy and quantitatively by SDS–PAGE. Using the CPP cocktail JBS-Proteoducin, the intracellular concentrations of a secondary antibody and bovine serum albumin can reach the micromolar range. Internalization of antibodies allows mediation of intracellular pathways including knock down of signal transduction. The high specificity and affinity of antibodies makes them potentially more powerful than siRNA. Thus, CPPs represent a significant new possibility to study signal transduction processes in competition or in comparison to the commonly used other techniques. To estimate the highest attainable intracellular concentrations of cargo proteins, the CPPs are tested for cytotoxicity. Cell viability and membrane integrity relative to concentration of CPPs are investigated. Viability as estimated by the reductive activity of mitochondria (MTT-test) is more sensitive to higher concentrations of CPPs versus membrane integrity, as measured by the release of dead cell protease. Distinct differences in uptake efficiency and cytotoxic effects are found using six different CPPs and six different adhesion and suspension cell lines. J. Cell. Biochem. 112: 3824–3833, 2011.

KEY WORDS: CELL PENETRATING PEPTIDES; NON-COVALENT COMPLEXES; INTERNALIZATION OF PROTEINS; INTRACELLULAR CONCENTRATION; CYTOTOXICITY

The plasma membrane protects the cell from its environment. Although normally permeable only by lipids and small non-polar molecules, various chemical or mechanical methods will allow transport across the membrane including; lipophilic and amphiphilic detergents, liposomes, viruses, virus particles, microinjection, electroporation to transfect cells with plasmids, genes, micro-RNA, oligonucleotides, and mimics of nucleic acids. Yet none of these methods efficiently transfect cells and/or require additional selection of clones. They are also generally not suitable for in vivo experiments, animal studies, or drug delivery to patients. Hence, other techniques are required to transport cargo through the plasma membranes of live cells.

Our focus in this work is the internalization of peptides and proteins into live cells. The intracellular delivery of information-rich molecules can modulate protein–protein interactions and thereby influence cellular mechanisms of different diseases. In contrast to transfection of cells with DNA or RNA, the above listed methods are not methods of choice for transduction of proteins into live cells. In the last two decades a number of peptides and proteins were shown to have cell penetrating properties [Snyder and Dowdy, 2005; Heitz et al., 2009; Lindgren and Langel, 2011]. These sequences are termed "*Trojan horse peptides*", "*Protein Transduction Domains" (PTDs)*, or "*Cell penetrating peptides*" (CPPs). Such peptides and proteins with a *membrane transduction domain* are derived as partial sequences from transcription factors, bacterial or viral surface proteins, toxins, amphiphatic helix-forming peptides and from ligands of membrane-bound receptors or adhesion proteins [Rennert et al., 2006; Partlow et al., 2008]. Different types of CPPs have different amino acid sequences, but all contain a transduction domain. They can be classified based on their structural properties. The most frequently used CPPs are penetratin [Dupont et al., 2011], HIV-Tat [Torchilin, 2008], polyArg (R_8) [Moulton et al.,

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2004], PEP-family peptides [Kurzawa et al., 2010; Crombez and Divita, 2011], substituted loligopeptides [Fischer et al., 2002; Kocznan et al., 2002], ligands for integrins [Partlow et al., 2008], for G-protein coupled receptors [Covic et al., 2002; Rennert et al., 2006], and for immune-receptors. They allow mediation of intracellular pathways including knock down of signal transduction by the use of antibodies. The high specificity and affinity of antibodies makes them potentially more powerful than siRNA or in the case of protein kinases, more selective than the typical ATP-derived inhibitors. Furthermore, CPPs allow the transport of antibiotics, enzymes, enzyme substrates and inhibitors, of ligands for protein binding domains, of toxins as well as peptide modulators and stabilisators of functional proteins.

Thus, CPPs represent a significant new possibility to study signal transduction processes in competition or in comparison to the commonly used other techniques. In particular internalization of cancer-fighting antibodies may support cancer therapy by blocking of tumor-specific protein–protein interactions [Bitler and Schroeder, 2010]. CPPs may be used as carriers for diagnostic purposes and for therapeutics including treatment of cancer, cardiological disease, pain, stroke and muscle dystrophy [Moulton et al., 2009; Rennert et al., 2009; Bitler and Schroeder, 2010; Delcroix and Riley, 2010; Olson et al., 2010; Johnson et al., 2011]. They can mediate transdermal delivery, too [Desai et al., 2010].

In contrast to the above mentioned compounds and methods for uptake of nucleic acids there are besides the cocktail JBS-Proteoducin only few compounds commercially available designed for protein delivery (Chariot™, ImmunoCellin™, ProteoJuice™, and PULSIN™).

The transport of the cargo through the plasma membrane has proven to be a complex process, and is not yet fully understood. The dynamic uptake process requires the interaction of the conjugate or complex, formed from the cargo and CPPs, with the membrane bound proteoglycans [Ziegler and Seelig, 2008] or/and membrane lipids [Henriques et al., 2010; Graeslund and Maeler, 2011], uses the microheterogenity of the cell membranes [Partlow et al., 2008] and occurs by different mechanisms [Zorko and Langel, 2005; Soldati and Schliwa, 2006; Fretz et al., 2007; Nakase et al., 2007]. It is believed that macropinocytosis is the primary mechanism causing internalization [Kaplan et al., 2005], leading to an endocytotic uptake. Peptides that have high affinity for membranes have a higher propensity to be internalized by a non-endocytic mechanism than peptides with a lower affinity [Henriques, 2010]. CPPs with low molecular weight cargos may also enter without vesicle formation and facilitate access to all intracellular compartments [Tuennemann and Cardoso, 2010].

The efficient delivery of cargo into live cells depends on particular factors such as the cell line, passage number, exposure and processing time, temperature, and concentrations of the cargo-CPP complex, and of auxiliary reagents [Richard et al., 2003; D'Souza-Schorey and Chavrier, 2006; Guerkan et al., 2006; Soldati and Schliwa, 2006; Nakase et al., 2007]. These parameters may have to be individually optimized.

The cargos can be covalently coupled to CPPs, as for example by fusion proteins or chemically prepared conjugates. We prefer internalization by the complexation of a cargo molecule with suitable CPPs [Gros et al., 2006; Morris et al., 2007; Meade and Dowdy, 2008; Heitz et al., 2009]. It is the most convenient method, requiring only a mixing of the components with subsequent formation of non-covalent aggregates. For transduction of peptides and proteins a 10- to 20-fold excess of CPP (molar ratio) is necessary. Cocktails of CPPs such as Proteoducin provide a universal approach for internalization of cargo through compatibility with numerous cell types containing various membrane structures, triggering different mechanisms of transduction and allowing complexation with structurally different cargos.

Mammalian cells can strongly differ in their protein, proteoglycan and lipid composition of membranes, and in their signal pathways. We used certain adhesion and suspension cell lines to compare their preferences for distinct CPPs, their uptake efficiencies and to check their viability and membrane integrity in the presence of increasing amounts of these CPPs. Estimation of the highest possible yield of internalized cargo amounts is a prerequisite for specific influencing of intracellular processes, for in vivo experiments and for therapeutic applications.

MATERIALS AND METHODS

CELL PENETRATING PEPTIDES AND COCKTAIL "JBS-PROTEODUCIN"

MPGα: AcGALFLAFLAAALSLMGLWSQPKKKRKV-NH-CH_2-CH_2-SH (five positive charges, MW 3,047 Da, Jena Bioscience GmbH),

MPGβ: AcGALFLGFLGAAGSTMGAWSQPKKKRKV-NH-CH_2-CH_2-SH (five positive charges, MW 2,910 Da, Jena Bioscience GmbH),

CAD-2: GLWRALWRLLRSLWRLLWKA-NH-CH_2-CH_2-SH (six positive charges, MW 2,653 Da, Jena Bioscience GmbH),

Penetratin: RGIKWFGNRRMKWKK (eight positive charges, MW 2,247 Da, Jena Bioscience GmbH),

HIV-Tat (47–57): YGRKKRRQRRR (nine positive charges, MW 1,560 Da, Jena Bioscience GmbH),

CPPP-2 (BAX inhibitory peptide): KLPVM (two positive charges, MW 605 Da, Jena Bioscience GmbH),

JBS-Proteoducin (cocktail of CPPs, Jena Bioscience GmbH).

CELL CULTURES

Adhesion cells. HeLa (Human cervic carcinoma), COS-7 (African green monkey kidney), NIH 3T3 (Swiss mouse embryo).

Suspension cells. Jurkat (Human T-cell leukemia), NB-4 (Human acute promyelocytic leukemia), Kasumi-1 (Human acute myeloid leukemia).

COMPLEX FORMATION

CPPs (0.5 mg), or the vial of JBS-Proteoducin, were dissolved in 1.25–1.50 ml of sterile and oxygen free water (bubble helium or argon through the water). To achieve with CPPP-2 the required 10 times higher molar ratio 1.2 mg was dissolved in 1 ml oxygen free water.

The solution was vortexed, frozen to −80°C and thawed three times, and sonicated for 5 min. The resulting stock solutions were used immediately or stored as aliquots at −20°C.

The cargo proteins (FITC-Ab, MW ∼150 kDa, ATTO488-BSA MW = 68 kDa) and 1 μl stock solution of CPPs or JBS-Proteoducin

were dissolved separately each in 100 μl of phosphate buffer (Dulbecos PBS 1×, without Ca and Mg, pH 7.0–7.5, PAA, The Cell Culture Company, Pasching, Austria). Both solutions were thoroughly mixed by repeated pipetting (6×). The mixture was incubated for 30 min at 37°C to achieve complex formation. The molar ratio of cargo to nucleotide should be calculated to 1:10. For internalization of higher amounts both the amount of protein and of stock solution of CPP or cocktail, respectively, were multiplied.

Complexes of the enzyme β-Galactosidase (E. coli, 540 kDa, Calbiochem, Darmstadt) and JBS-Proteoducin were formed from 1 μg β-Galactosidase and 1 μl stock solution of JBS-Proteoducin under the described conditions.

INTERNALIZATION

Because the transduction protocol was calculated for six-well/35 mm culture plates, the volumes of media for other vessels needed were adjusted accordingly. Cells were cultivated and transduced under the commonly used optimum conditions. Only low passage numbers were used and the cells were checked microscopically for their vital shape, the absence of bacteria, and also for the absence of mycoplasma by PCR. A solution of penicillin and streptomycin was added to prevent growth of bacteria during incubation.

Adherent cells. Medium was thoroughly aspirated from prepared cells and the cells were washed three times with PBS (Tris, PUFFERAN®, Carl Roth, Karlsruhe) at 37°C. Two hundred microliter of complex solution, followed by 400 μl of serum free medium, were added. Incubation was started under gentle mixing for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. Then 1 ml of complete growth medium was added and incubation continued at 37°C for approximately 20 h in a humidified atmosphere containing 5% CO_2. The cells were washed twice with PBS, three times with glycine buffer (Glycine buffer, pH 3, 200 mM, Jena Bioscience GmbH) and twice with PBS (2 ml each).

Suspension cells. The thoroughly washed cell pellet was suspended in 200 μl of complex solution followed by 400 μl of serum free medium. Incubation was started under gentle mixing for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After addition of 1 ml of complete growth medium, incubation was continued at 37°C for approximately 20 h in a humidified atmosphere containing 5% CO_2. The cells were washed by spinning down twice with PBS, three times with glycine buffer pH 3 and twice with PBS (2 ml each).

INTERNALIZATION OF β-GALACTOSIDASE

Cells were grown on a cover slip (0.15×10^6 cells per well). Cover slips with a modified surface (poly-lysine) were used for adhesion of suspension cells. The slip was placed into a well before growing the adherent cells or before transferring the transduced suspension cells.

To the serum-free incubation medium 10% dimethyl sulfoxide (DMSO 99.5% for molecular biology, Carl Roth, Karlsruhe) was added. After incubation with the complete growth medium the cells were thoroughly washed and stained with X-Gal (99%, Calbiochem). The cover slip was placed on a slide and the cells were directly observed without fixation (Microscope, Nikon eclipse TS100, Nikon, Japan). A magnification of 100× was used.

QUALITATIVE DETECTION OF INTERNALIZED CARGO BY FLUORESCENCE MICROSCOPY

Cells were grown on a cover slip (0.15×10^6 cells per well) and incubated as described above, but without DMSO in the serum-free transduction medium. After incubation the cover slip was placed on a slide and the fluorescence directly observed without fixation (Fluorescence microscope, Axiophot, Carl Zeiss, Oberkochen, Germany). A magnification of >100× was used.

QUANTITATIVE ESTIMATION OF INTERNALIZED FLUORESCENT CARGOS BY SDS–PAGE

HeLa-cells were transduced with a FITC conjugated polyclonal secondary antibody goat against mouse under standard conditions for adherent cells (1 h at 37°C, serum free medium, containing 10% DMSO and 20 h at 37°C with complete growth medium). The transduced and thoroughly (three times) washed cells (0.3×10^6) from a six-well plate were treated for 10 min at 37°C with 0.7 μl trypsin/EDTA solution (Trypsin/EDTA, 1×, concentrate, PAA, The Cell Culture Company, Pasching, Österreich) per well. After addition of 0.7 ml phosphate buffer (PBS) the cells were separated by spinning down for 5 min. at 5,000 rpm (Table centrifuge "Eppendorf Centrifuge 5424"). To each tube 5 μl Roti®-Load solution (Protein-Gel loading buffer, Roti®-Load 2, non reductive, 4× conc. Carl Roth, Darmstadt) was added, treated with ultrasound for 5 min at 85°C and the cells separated by spinning down for 5 min at 1,000 rpm. The same procedure was performed with the standard (FITC conjugated antibody, diluted with 15 μl water and 5 μl Roti®-Load solution). Twenty microliter of each probe were applied to a SDS gel (10%), analyzed by electrophoresis (Vertikal Mini Polyacrylamide Gel System, Biorad Munich, Germany) and detected with a digital fluorescence densitometer (UV Transimulator and CCD Video Camera Modul, LTF Labortechnik, Wasserburg, Germany). Fluorescence intensities were compared to the standard (0.02, 0.1, 0.2, 0.4, 0.6, and 1.0 μg FITC coupled secondary antibody, molecular weight 1.5×10^5 Da).

CALCULATION OF INTRACELLULAR CARGO CONCENTRATION

The diameter of a HeLa-cell was estimated from fluorescent microscopic pictures using a calibration scale. The mean volume was calculated to 1.2×10^{-5} μl. SDS–PAGE with fluorescent proteins allows the calculation of the internalized amount in one cell. This amount divided by the mean cell volume provides the intracellular concentration.

USE OF AUXILIARY COMPOUNDS

To enhance transduction efficiency and for high yield transduction, certain auxiliary compounds can be used. CPPs and their cocktails can be degraded by secreted or by membrane-bound enzymes. To reduce this inactivation before internalization BSA, aprotinin, *o*-phenanthrolin, and other protease inhibitors can be added to the serum free incubation medium. In the case of MPGα, MPGβ, CAD-2 and the cocktail JBS-Proteoducin, the addition of SH-reactive inhibitors should be avoided. BSA acts as a co-substrate for extra and intracellular proteases and enhances the cargo uptake into cancer cells as a cell food [Qu et al., 2010].

DMSO, added after complex formation to the transduction medium, enhances the permeability of cell membranes [Wang et al., 2010]. To release the cargo from intracellular vesicles, the addition of destabilizers such as chloroquine [Shiraishi et al., 2005], wortmannin [D'Souza-Schorey and Chavrier, 2006; Guerkan et al., 2006; Soldati and Schliwa, 2006], and Ca-ions [Shiraishi et al., 2005] is recommended after finishing the transduction process. These compounds are given only to the complete medium.

VIABILITY ASSAY

The assay (MTT-Test, Jena Bioscience GmbH, Germany) was performed according to the instructions. Briefly, cells were cultivated under commonly used conditions and treated in serum-free medium for 1 h at 37°C with different CPPs or with the cocktail. After removal of CPPs and repeated washings, the MTT-test was used to estimate cell viability. Untreated cells were defined as 100% viable. The assay is performed according to the instructions in six-well plates. Each value is estimated as a triplicate with a SD of ±1.3. The formed formazan was measured with an UV/VIS spectrometer (Lambda 35, Perkin-Elmer, Waltham) at 508 nm.

MEMBRANE INTEGRITY ASSAY

Cells were cultivated under commonly used conditions and treated in serum-free medium for 1 h at 37°C with different CPPs and the cocktail. After removal of CPPs and repeated washings, the integrity of the cell membrane was estimated by the bioluminescence test ("CytoTox-Glo™", Cytotoxicity Assay, Promega, Madison) measuring the release of a cytosolic protease. The Cyto-Tox-Glo™ assay was performed according to the instructions and adapted to 96-well plates. Bioluminescence is estimated by a Luminescence plate reader (Fluostar OPTIMA, BMG Labtech, Offenburg, Germany). Each value is estimated as a triplicate with a SD of ±1.2.

MYCOPLASMA TEST

The test (Mycoplasma Detection Kit, Jena Bioscience GmbH) was performed according to the instructions. This PCR test is ultrasensitive, contamination at the beginning stages can be detected.

RESULTS AND DISCUSSION

INTERNALIZATION OF ENZYMES

Different cell lines were transfected with the enzyme β-Galactosidase. The microscopic pictures demonstrate that the cocktail JBS-Proteoducin transported the high molecular weight enzyme (540 kDa) into all tested adhesion and suspension cell lines, including the transfection-resistant Kasumi-1 cells (Fig. 1). The cells were stained with X-Gal, indicating that functionally active β-Galactosidase was transported into all cells. Distinct cells were stained to different intensities, no cell was left unstained The total number of cells in the field of view differed depending on adhesion at the surface of the cover slip and on the detachment of cells by the washing procedures, in particular the suspension cells. The complex formation with the positively charged CPPs is enforced by the negative charges of the surface of β-galactosidase. The enzyme

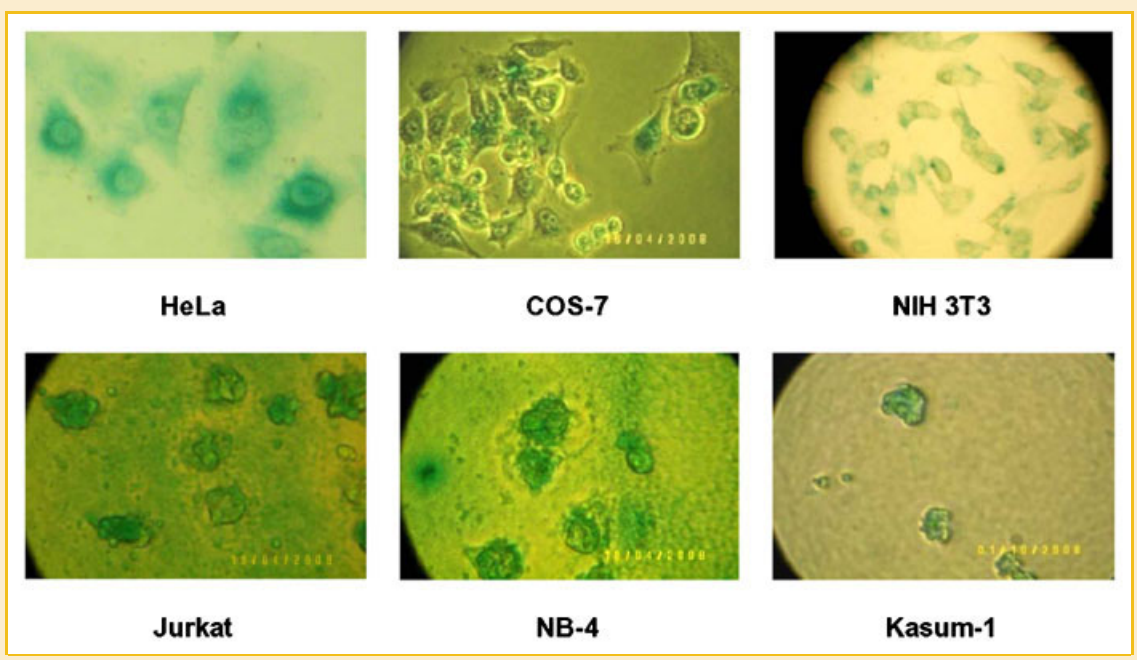

Fig. 1. Internalization of the enzyme β-galactosidase into different adhesion and suspension cells: Non-covalent complexes of β-galactosidase (540 kDa) and JBS-Proteoducin were formed and under the described conditions transported into different cell lines. The product of the intracellular enzymatic reaction was colored with X-Gal. All cells even including the transfection-resistant Kasumi-1 cells are stained, but with different intensities. The total number of cells in the microscopic field of view depends on the adhesion on the surface on the cover slip. Thorough wash procedures can remove suspension cells from the cover slip. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

reaction increases the number of stained molecules, thus this test is optimal for preliminary studies on uptake conditions.

In the literature described internalization of other enzymes, like Cre-recombinase and lytic enzymes [Nagel et al., 2008; Gitton et al., 2009; Borysowski and Gorski, 2010; Dietz, 2010], demonstrate the potential of CPPs in the field of therapeutic use of enzymes.

INTERNALIZATION OF ANTIBODIES AND BOVINE SERUM ALBUMIN

Qualitative estimation of uptake of fluorescent proteins (BSA and antibodies). Fluorescence microscopy allows not only to detect fluorescent cells but also to estimate the intensity of fluorescence, thereby allowing an investigation into the influence of CPPs and different incubation conditions which affect uptake efficiency. Figure 2 shows that ATTO488-labeled bovine serum albumin (ATTO-BSA, Jena Bioscience GmbH) is transported very efficiently into the HeLa cells. A comparison of the control versus fluorescence images shows that all cells are fluorescent. The intensity of fluorescence depends on: The concentration of the complex formed with MPGα, the temperature (4 and 37°C), and incubation time (1, 4, or 6 h). It should be noted that in the case of labeled BSA, internalization can be detected to a lesser degree also without MPGα. This might be a result of the observed uptake of BSA by "hungry" cancer cells [Ward et al., 2000; Qu et al., 2010].

We internalized a monoclonal (monoclonal anti-actin antibody ATTO488 conjugate, labeled by Jena Bioscience, Germany) as well as two polyclonal fluorescent labeled antibodies (polyclonal antibody goat anti-mouse Ig's, Fluoresceine conjugate, Biosource, Camarillo and polyclonal PI3-kinase gamma antibody, ATTO488 conjugate, provided by Jena Bioscience) into HeLa and Jurkat cells. These antibodies were efficiently internalized, depending on the concentration of the complex in the incubation medium, temperature, and time. The monoclonal antibody with a high affinity to actin was able to label intracellular structures (cytoskeleton) very well. HeLa cells can show, at higher magnification, the intracellular distribution of the labeled antibodies as well as indicate the cytotoxic effects of high concentrations by changing their cell shape.

For the used CPPs the following rank order for internalization of proteins (BSA and polyclonal antibodies) were found: CAD-2 > MPGα = MPGβ ≫ CPPP-2. With penetratin and HIV-Tat no detectable amounts could be internalized by formation of non-covalent complexes. For the uptake of nucleoside triphosphates we found another rank order [Mussbach et al., 2011]. It should be mentioned that the CPPs also differ in their hydrophobicity and stability against proteolytic degradation. CAD-2 is the most hydrophobic and most stable compound. In the presence of COS-7 and NB-4 cells it remains completely intact for 60 min. Determination of structure activity relationships for uptake efficiencies of CPPs needs to consider the different behavior of the cell lines. It requires knowledge of three-dimensional structure, flexibility and topological distribution of positive charges, and hydrophobic residues of the CPPs under physiological conditions. Till now it is difficult to estimate such relationships, even for one cell line.

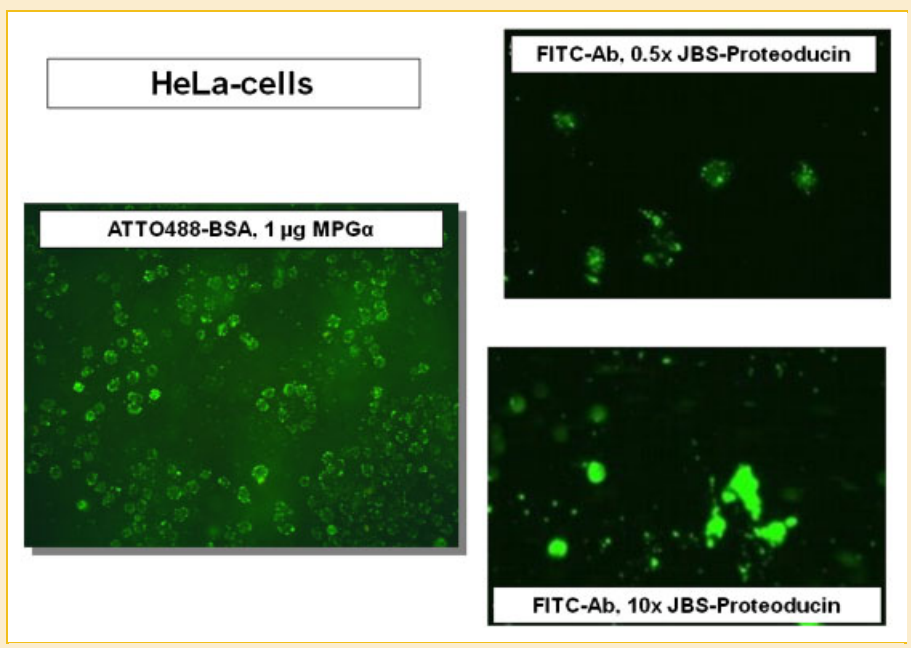

Fig. 2. Qualitative detection of uptake of fluorescent labeled proteins by fluorescence microscopy: ATTO488-labeled bovine serum albumin (ATTO-BSA) and FITC-conjugated secondary antibody goat against mouse (FITC-Ab) are transported as non-covalent complexes with MPGα and JBS-Proteoducin into HeLa-cells. The fluorescence microscopic pictures show that all cells are fluorescent, despite intensive wash procedures. The fluorescence is not homogeneously distributed over the whole cell but seems to be localized in vesicles. The intensity of the fluorescence depends on concentration of the complex, temperature, and incubation time. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Reached intracellular amounts and concentrations. In the literature, uptake was quantitatively evaluated by MALDI-TOF mass spectrometry [Palm et al., 2006] and the fluorescent CPPs by HPLC [Aussedat et al., 2006].

We made attempts to quantify the internalized amount of labeled and unlabeled antibodies and of BSA. The cell lines were incubated with the CPP-cargo complexes under the described conditions. After thorough washings and cell lyses the antibodies were detected, in the case of the primary antibody, against PI3-kinase γ with a secondary peroxidase-linked antibody, or by fluorescent (ATTO488 or FITC) labeled antibodies by fluorescence detection after electrophoretic separation. To quantify the internalized amounts, the fluorescence intensities were compared to standard amounts of labeled antibodies. Figure 3 shows that the antibody is partially divided into light and heavy chains under the used conditions. Detectable fluorescence was only attainable with an extracellular concentration of 5 μg/1.6 ml fluorescent antibody complexed with a fivefold amount of JBS-Proteoducin. About 0.4–1.5% of the extracellular amount of antibody could be internalized. Considering the cell number per well (0.3×10^6) the uptake per cell was calculated to 4.3 amol. This result correlates well with uptake experiments with the primary antibody against PI3-kinase γ. Thus, internalization without electrophoretic migration also produced nearly the same results.

To verify the results obtained with antibodies, the uptake of bovine serum albumin conjugated with ATTO488 was estimated using the same procedure. The results correlate with that obtained for the FITC conjugated secondary antibody. However, BSA might be considered a special type of cargo as it acts as a nutrient for the cells. This particular function could explain the relatively substantial internalized amount of 50 amol per cell and intracellular concentration of 4.3 μM which was achieved. Nevertheless, fluorescent BSA is only detectable beginning at a 10-fold extracellular amount of the complex, compared to the commonly used minimum concentration, used for microscopic detection.

Table I summarizes the results obtained with different cargos and indicates that BSA as cargo produces the best results. The internalized amounts increase with an increased concentration in the extracellular incubation medium, in the case of BSA even linearly. Knowledge of attainable intracellular amounts of antibodies is necessary to calculate the conditions for a complete inactivation of functional proteins. This theoretical calculation also requires the complete release of the antibody from endosomes, which can be supported by auxiliaries. Taking the mean cell volume of HeLa-cells also the intracellular concentration can be estimated. We calculated the attainable amount of secondary antibody per cell to 4.3 amol, leading to an intracellular concentration of 0.6 μM. This concentration can be reached because the cocktail JBS-Proteoducin, optimized for internalization of proteins, even at 15 times higher concentration (15 μl stock solution) does not reduce cell viability nor membrane integrity.

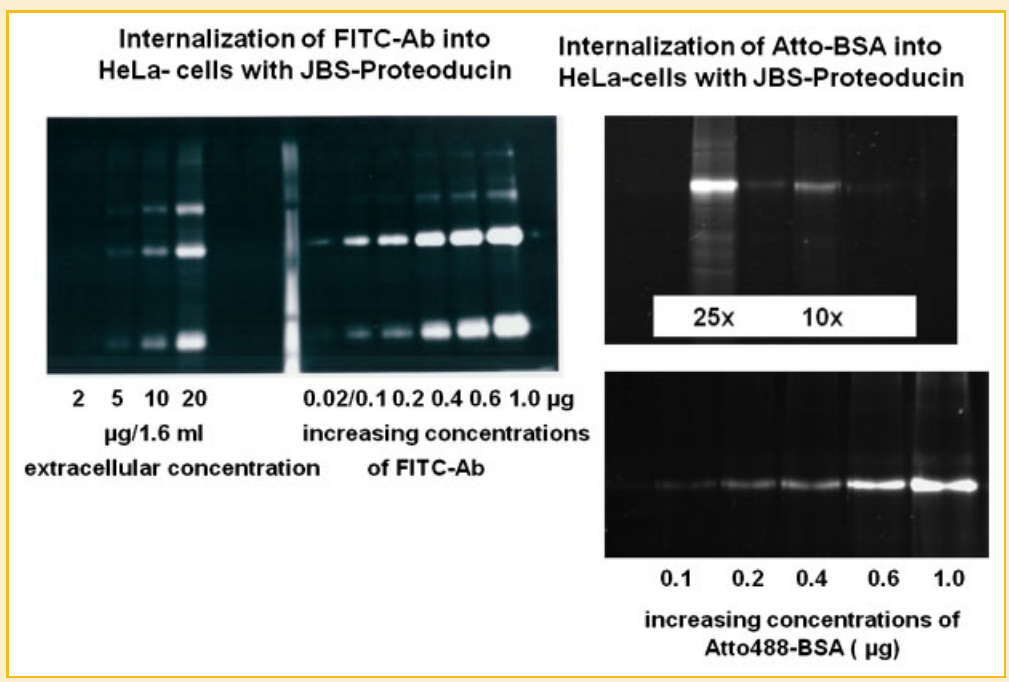

Fig. 3. Quantitative estimation of internalized amount of ATTO488-labeled bovine serum albumin (ATTO-BSA) and of a FITC-conjugated secondary antibody coat against mouse (FITC-Ab) by SDS–PAGE: The antibody complexed with JBS-Proteoducin is internalized in increasing concentrations into HeLa-cells under standard conditions (37°C, 1 + 20 h). In a similar procedure increasing concentrations of ATTO-BSA complexed with JBS-Proteoducin are internalized. After thorough washing, cell lyses and centrifugation the homogenate is analyzed by gel electrophoresis. The fluorescence intensities of the electrophoretic bands are compared to standard concentrations of ATTO-BSA and of FITC-Ab, treated under the same conditions. About 4% of the extracellular amount is internalized. Under the used conditions the antibody is partially divided into heavy and light chains. The internalized amount reaches 0.4–1.5% of the antibody amount in the incubation medium.

TABLE I. Quantitative Estimation of Uptake Efficiency of Non-Covalent Complexes Formed From Fluorescent Proteins and JBS-Proteoducin

Amount of added complex (per 1.6 ml serum-free medium)	Internalized amount (0.3×10^6 HeLa-cells per well)	
	Percentage of extra-cellular amount	amol per cell
ATTO448-Bovine serum albumin		
10 μg + 10 μl JBS-Proteoducin	4	20
25 μg + 25 μl JBS-Proteoducin	4	50(≈4.3 μM)
FITC-Antibody (secondary, polyclonal, goat anti mouse lg's)		
5 μg + 3.75 μl JBS-Proteoducin	0.4	0.4
10 μg + 7.5 μl JBS-Proteoducin	0.5	2.2
20 μg + 15 μl JBS-Proteoducin	1.5	4.3(≈0.6 μM)

Into HeLa-cells internalized amounts of ATTO488 labeled bovine serum albumin and FITC-conjugated secondary antibody are calculated from fluorescence intensities taken from SDS–PAGE (Fig. 3). The intracellular amounts are calculated from total internalized amounts divided by the number of cells in one well, the intracellular concentrations by division with the cell volume. The attainable intracellular amounts and concentrations are without cytotoxic effects.

CYTOTOXIC EFFECTS OF CPPS

As cytotoxic effects of CPPs may negatively affect transduction experiments, critical observation of cell viability [Wu et al., 2007], and/or cell membrane integrity [Niles et al., 2007; Bauer et al., 2006] is necessary to identify toxicity issues. Mainly the internalization of large amounts is limited by the concentration-dependent toxicity of CPPs. To estimate the attainable optimum concentration for cargo internalization we studied the influence of CPPs, cocktails, and auxiliaries on both cell viability and membrane integrity, respectively.

Influence on cell viability. Cell viability was tested with the MTT-test. This test is based on the activity of mitochondria, which can reduce the formazan to a purple colored compound measured at its absorption maximum at 508 nm. Figure 4 demonstrates that the influence on cell viability is strongly concentration-dependent. Up to concentrations of 10 μM, the influence of MPGα on the viability of HeLa-cells is only marginal. This concentration is sufficient to complex, in the incubation medium, 100 μg/ml of a protein with a MW of about 100 kDa. Surprisingly, MPGα at concentrations higher than 10 μM significantly reduces cell viability.

In a concentration of 8.2 μM, MPGα is not only non-toxic for HeLa-cells, but also has no toxic effects on the other cell lines (Fig. 5). MPGα causes an increase in viability of COS-7 and NIH 3T3 cells of more than 300%. We suggest that this strong effect results from an influence on the signal pathways.

Also other CPPs than MPGα at concentrations up to 10 μM only marginally influence the viability of HeLa or Kasumi-1 cells. Thus, MPGβ and Penetratin reduce the viability only slightly. TAT, CAD-2, and CPPP-2 are even able to enhance the mitochondrial dehydrogenase activity leading to an increased amount of the formazan compound (Fig. 6). This type of enhancement might result from the stabilization of mitochondria or from cell proliferation, stimulated by the CPPs. The BAX inhibitory peptide CPPP-2 is applied in a 10 times higher concentration than the other CPPs. Due to the cytoprotective activity of CPPP-2 [Gomez et al., 2007; Gomez and Matsuyama, 2011] the strong increase in cell viability is not surprising.

Auxiliary compounds. Auxiliary compounds such as permeability enhancers, protease inhibitors and vesicle destabilizers may improve the transduction process. Their influence on cell viability depends not only on their concentration but also on the cell line used. Millimolar concentrations of Ca^{2+} strongly reduced viability while wortmannin and chloroquine were tolerated much better by all six cell lines. In the case of COS-7 and NIH 3T3-cells the viability was enhanced by wortmannin and chloroquine from 150 to 250%, indicating that both compounds seem to influence intracellular

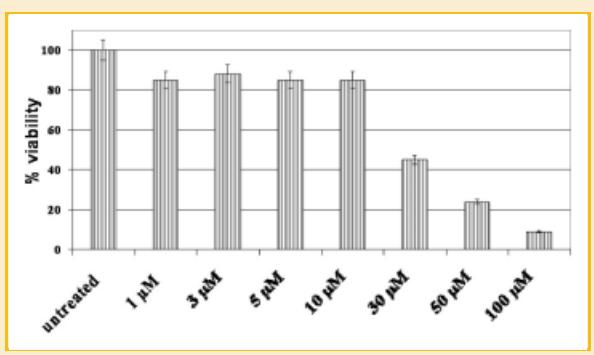

Fig. 4. Influence of increasing concentrations of MPGα on the viability of HeLa-cells: HeLa-cells are cultivated under commonly used conditions and treated in serum-free medium for 1 h at 37°C with increasing concentrations of MPGα. After removal of the MPGα and repeated washings the viability is estimated by the MTT-test. Untreated cells are defined as 100% viable. In concentrations up to 10 μM MPGα has only a marginal influence on the viability of HeLa-cells. But in higher concentrations the viability is strongly reduced.

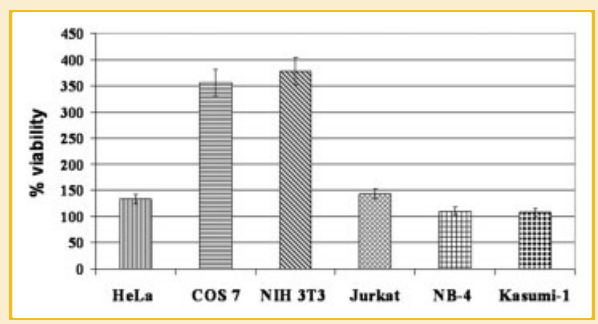

Fig. 5. Influence of MPGα on the viability of different cell lines: Different adhesion and suspension cells are treated under commonly used conditions with 8.2 μM concentrations of MPGα. The viability is compared to untreated cells. As expected most cell lines show only marginal influence on viability. COS-7- and NIH 3T3-cells show a viability which is enhanced to more than 300%.

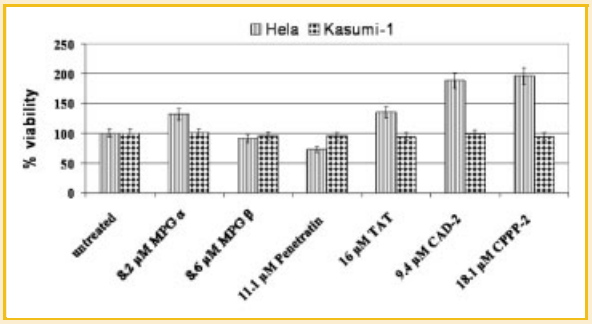

Fig. 6. Influence of different CPPs on the viability of HeLa- and Kasumi-1 cells: HeLa- and Kasumi-1 cells are cultivated and treated under commonly used conditions with MPGα, MPGβ, CAD-2, penetratin, HIV-Tat, and CPPP-2 in the indicated concentrations. Since MPGα and MPGβ have only marginal influence on the viability of HeLa-cells, penetratin reduces the viability slightly. CAD-2, HIV-Tat and CPPP-2 are able to enhance the viability. The viability of Kasumi-1 cells is considerably less sensitive to all applied CPPs, even number enhancement can be observed.

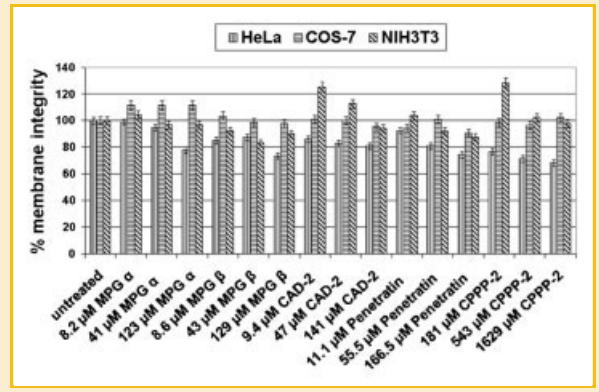

Fig. 7. Influence of different CPPs on the membrane integrity of HeLa-, COS-7- and NIH3T3-cells: The cells are cultivated and treated under commonly used conditions with CPPs. Membrane integrity was estimated by the bioluminescence test "CytoTox-Glo™" measuring the release of a cytosolic protease. The CPPs are applied in higher concentrations than in the MTT-test. HeLa-cells are more susceptible to all tested CPPs than the other two cell lines.

signal pathways. It should be noted that the inhibitor wortmannin influences the PI3-kinase mediated signal transduction.

BSA (0.5%) and DMSO (10%) did not adversely affect viability although 30% of the DMSO was excluded from the internalization experiments. The application of auxiliaries also requires checking their cytotoxicity [for details see: Mussbach et al., 2011].

Influence on membrane integrity. Membrane integrity of the adhesion cells HeLa, COS-7, and NIH3T3 is only slightly influenced by the most commonly used CPPs, even at higher concentrations (Fig. 7). At a more than 10-fold higher concentration than applied for the MTT-test, the membrane integrity is only slightly reduced for COS-7 and NIH3T3-cells. Highest tested and in this test non-toxic concentrations come to a range from 123 to 141 μM for MPGα, MPGβ, and CAD-2, to 160 μM for penetratin and to 1,600 μM for CPPP-2. Even at a more than 10-fold higher concentration, compared to viability measurements, the membrane integrity is only slightly influenced. The membrane permeability of HeLa-cells seems to be more sensitive to the CPPs used in this investigation compared to the other two suspension cell lines. The seeming enhancement of membrane integrity in COS-7 and NIH3T3 cells by MPGα and CAD-2 can hypothetically result from different mechanisms in the whole procedure of formation of the bioluminescent end product; most probably it can result from the promiscuity of peptides and proteases. But, there exist no experimental studies about this effect.

Cytotoxicity of JBS-proteoducin. The impact of the cocktail JBS-Proteoducin on cell viability was systematically investigated for six cell lines (Fig. 8). Most cell lines tolerated treatment with the cocktail very well, even at high concentrations. Thus, at levels 15 times higher than recommended only marginal effects on cell viability were observed. Only the Jurkat cells were to some degree susceptible at this high concentration.

Membrane integrity: In contrast to the single CPPs some cell lines were even sensitive to the normal concentration of the cocktail JBS-Proteoducin. Especially the NB-4 cells were susceptible to the cocktail. Other cell lines, including HeLa-, COS-7 and Kasumi-1 tolerate concentrations up to 15 times higher. Compared to other used cell lines Kasumi-1 seems to be in both cytotoxicity tests the most insensitive cell line against used CPPs. These findings demonstrate that the cytotoxicity depends not only on the

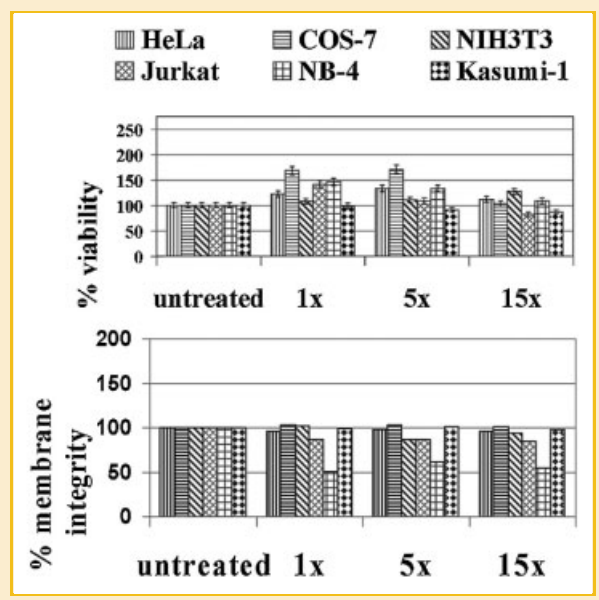

Fig. 8. Influence of increasing concentrations of JBS-Proteoducin on the viability and membrane integrity of different adhesion and suspension cell lines: The cell lines were cultivated and treated under commonly used conditions in serum free medium for 1 h at 37°C with increasing concentrations (1 µl = 1×, 5 µl = 5×, and 15 µl = 15×) stock solution of the cocktail JBS-Proteoducin in 600 µl serum free incubation medium. *Viability*: In the recommended concentration JBS-Proteoducin does not reduce the viability of any cell line. Most cell lines also tolerate 5–15× higher concentrations very well. Only Jurkat and Kasumi-1 cells are to some degree susceptible to high concentrations of JBS-Proteoducin. *Membrane integrity*: Some cell lines are even sensitive to the normal concentration of JBS-Proteoducin, especially the NB-4 cells. Other cell lines, including HeLa-, COS-7 and Kasumi-1 are able to tolerate concentrations up to 15 times higher.

concentration, but also on the type of CPP, on the composition of the cocktail as well as on the cell line.

The strong differences found between both cytotoxicity tests result from different mechanisms, including the reductive activity of mitochondria (MTT-test), as well as on the integrity of the cell membrane. Our FACS-analysis with propidium iodide (not shown here) agrees with the bioluminescence-test for membrane integrity. To avoid false or misleading results due to working with damaged cells, we recommend the use of low concentrations, and to test each cell line individually as the sensitivity of the cells vary according to CPP, cocktail, or the complexes formed with the cargo.

CONCLUSIONS

This study confirmed that CPPs as well as the cocktail JB-S-Proteoducin are able to transport peptides and proteins into live cells. Our attempts to find structure activity relationships between structure of CPPs, uptake efficiency and cytotoxicity were strongly complicated by the pronounced differences between the cell lines. To succeed in these studies more detailed knowledge is necessary about bioactive conformation of CPPs and of biology and biochemistry of the used cells. The intracellular amounts of antibodies and of BSA can reach micromolar concentrations. Such amounts are in some cases required for an adequate influencing of intracellular targets. Internalization efficiency depends not only on cell type, but also on the type of CPP, on the particular cargo, and on conditions such as passage number, incubation time, and temperature. Internalized antibodies act more specifically than inhibitors or mono-valent ligands for protein binding domains and can compete with silencing RNA-techniques. The formation of non-covalent complexes between cargo and CPP is a very convenient procedure. Yet the formation of covalent bonds in conjugates or fusion proteins allows higher concentrations of salts and other extracellular compounds in the incubation medium. But this method requires the intracellular cleavage of the conjugate. In both cases the release of the cargo from intracellular vesicles (endosomes) should be triggered by vesicle destabilizers. CPPs, cocktails and auxiliaries can influence membrane properties; they can affect the signal transduction, intracellular signaling, and membrane processes. Thus, they can cause toxic effects which are dependent on concentrations, cell line, cargo, and CPP. Anti tumor-, anti viral-, anti microbial- and signal transduction therapy require optimization of each application regarding CPP-type, concentrations and addition of auxiliaries. To avoid unspecific side effects a topical application is presently recommended. More investigative studies are required to fill in informational gaps surrounding the complex process of transporting cargo through the plasma membrane.

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