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Journal of Cellular Biochemistry 115:243–252 (2014)

Journal of Cellular Biochemistry

# Transduction of Proteins into *Leishmania Tarentolae* by Formation of Non-Covalent Complexes With Cell-Penetrating Peptides

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## ABSTRACT

Cell-penetrating peptides (CPPs) are used to transport peptides, proteins, different types of ribonucleic acids (or mimics of these molecules), and DNA into live cells, both plant and mammalian. Leishmania belongs to the class of protozoa having, in comparison to mammalian cells, a different lipid composition of the membrane, proteoglycans on the surface, and signal pathways. We investigated the uptake of two different and easily detectable proteins into the non-pathogenic strain *Leishmania tarentolae*. From the large number of CPPs available, six and a histone were chosen specifically for their ability to form non-covalent complexes. For *Leishmania* we used the enzyme β-galactosidase and fluorescent labeled bovine serum albumin as cargoes. The results are compared to similar internalization studies using mammalian cells [Mussbach et al., 2011a]. *Leishmania* cells can degrade CPPs by a secreted and membrane-bound chymotrypsin-like protease. Both cargo proteins were internalized with sufficient efficiency and achieved intramolecular concentrations similar to mammalian cells. The transport efficiencies of the CPPs differed from each other, and showed a different rank order for both cargoes. The intracellular distribution of fluorescent-labeled bovine serum albumin showed highest concentrations in the nucleus and kinetoplast. *Leishmania* are susceptible to high concentrations of some CPPs, although comparably dissimilar to mammalian cells. MPG-peptides are more cytotoxic in *Leishmania* than in mammalian cells, acting as antimicrobial peptides. Our results contribute to a better understanding of molecular interactions in *Leishmania* cells and possibly to new treatments of leishmaniasis. J. Cell. Biochem. 115: 243–252, 2014. 

**KEY WORDS:** CELL-PENETRATING PEPTIDES; LEISHMANIA TARENTOLAE, FORMATION OF NON-COVALENT COMPLEXES; INTERNALIZATION OF PROTEINS; INTRACELLULAR CONCENTRATIONS; INTRACELLULAR DISTRIBUTION; CYTOTOXICITY; UPTAKE EFFICIENCY

The species *Leishmania tarentolae* is a member of the family of *Trypanosomatidae* which belongs to the order *Kinetoplastida* and the phylum *Euglenozoa* of protozoa. *Leishmania* are unicellular eukaryotic microorganisms containing one large mitochondrion which provides the energy for a singular flagellum. The mitochondrion harbors the kinetoplast, a unique dense granular structure containing circular DNA. *Kinetoplastida* have drawn the attention of researchers for two main reasons: Firstly, they are known for unique solutions to general molecular biologic processes such as mRNA editing [Simpson et al., 2000; Ramírez et al., 2011] and polycistronic transcription [Clayton and Shapira, 2007]. Protein coding genes are constitutively transcribed into large polycistronic RNAs of several hundred kb which are then processed to individual mRNAs by a coordinated process of 5′-trans-splicing and 3′-polyadenylation [LeBowitz et al., 1993; Guenzl, 2010]. These and other properties make non-pathogenic *Leishmania* strains especially suitable as hosts for recombinant expression of glycosylated proteins [LEXSY].

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Manuscript Received: 12 April 2013; Manuscript Accepted: 14 August 2013
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 22 August 2013
DOI 10.1002/jcb.24654 • © 2013 Wiley Periodicals, Inc.

Secondly, many species of *Kinetoplastida* cause severe human and animal diseases of medical, veterinary, or agricultural impact. *Kinetoplastida* are parasites which colonize a broad range of hosts including vertebrates, insects, and plants. About 21 of the known 30 species of *Leishmania* cause human infections ranging from self-healing cutaneous to fatal visceral leishmaniasis [Banuls et al., 2007]. According to the World Health Organization (WHO) more than 12 million people in 88 countries are affected by leishmaniasis with about 2 million new cases per year and more than 350 million people at risk. Mortality is estimated as between 20,000 and 40,000 leishmaniasis deaths per year [Desjeux, 2004; Alvar et al., 2012]. Leishmaniasis occurs mainly in tropical regions and is transmitted to the vertebrate host by the bite of a sandfly. *Leishmania* exist in two morphologically distinct forms. In the gut and esophagus of the sandfly vector they live as flagellated promastigotes (14–20 μm in size). Through the bite of infected sandflies they are transmitted to the mammalian host and are phagocytosed by macrophages where they then transform into non-flagellated amastigotes (2–4 μm in size).

The promastigote form of *Leishmania* can be cultured in vitro in complex or defined media and over the last decade the non-pathogenic species *L. tarentolae* has been developed into a widely used recombinant protein expression system [Breitling et al., 2002; Kushnir et al., 2005, 2011]. The genomes of several *Kinetoplastida* have been sequenced [Aslett et al., 2010; TriTrypDB, 2013] as well as recently the *L. tarentolae* genome [Raymond et al., 2012]. Due to the recently revealed lack of genes involved in vesicular-mediated protein transport it might be possible that *L. tarentolae* is deficient in endosomal trafficking, thus limiting the use of endosomal uptake pathways.

Finding suitable targets useful for anti-parasitic drug development has become an important aim. Transduction studies of *L. tarentolae* can reveal the signal and metabolic pathways of these cells, to determine whether their characteristics are markedly different from similarities as well as dissimilarities to the cells of mammalian hosts. The goal of these studies is to perform the transduction of protozoa′s with non-covalent complexes of cargo proteins and cell-penetrating peptides (CPPs). The advantages of this method include easy handling, protection of the cargo against enzymatic degradation by an excess of CPPs, and intracellular release of the cargo without the necessity to selectively cleave a covalent linkage.

These studies are to some degree also a prerequisite for successful transfection of *Leishmania* cells in culture, which until now has only been performed using the very inefficient method of electroporation. Thus the reliable transduction procedure as well as the estimated cytotoxicities of CPPs may help in finding conditions for transfection with plasmids. But this task is by our experience quite more difficult than the transfection of *Leishmania* and is beyond the scope of our study.

The transport of cargoes into live cells is a complex process [Foerg et al., 2005; Nakase et al., 2007; Deshayes et al., 2010; Madani et al., 2011]. To remain within the focus of the study we chose to investigate only specific steps. The formation of non-covalent complexes between different cargoes and different CPPs is triggered by an optimum release of free enthalpy. Because CPPs differ in their sequence, in the distribution of charged and hydrophobic side chains, and in their conformation and conformational flexibility, each type of cargo prefers another CPP. The formed complexes with their different surfaces interact with proteoglycans and phospholipids of the cell membrane and trigger different uptake mechanisms [Foerg et al., 2005; Madani et al., 2011]. In our studies we used two different proteins as cargoes and seven different CPPs to measure cargo internalization and intracellular distribution both qualitatively and quantitatively. Due to their protozoan character *Leishmania* differ in their membrane properties [McConville and Ferguson, 1993; Schneider et al., 1994; Bradford et al., 1995, 2002; Jaffe and Dwyer, 2003; Raymond et al., 2012], preferred signal pathways [Nandan and Reiner, 2005; Algranati et al., 2006; Abu-Dayyeh et al., 2008], and possibly also uptake mechanisms from those of mammalian cells. Therefore we were interested in the comparison of our results to those obtained from previous investigations using six different mammalian suspension and adhesion cells [Mussbach et al., 2011a,b].

## MATERIALS AND METHODS

### CELL-PENETRATING PEPTIDES

MPGα, MPGβ, CAD-2, penetratin, HIV-Tat (47–57), CPPP-2, and auxiliaries (bovine serum albumin, BSA) were obtained from Jena Bioscience (Jena, Germany), histone type II-AS from calf thymus was from Sigma–Aldrich (Taufkirchen, Germany). Chemical characteristics of the used CPPs are listed in Table I.

### CARGOES

Fluorescent-labeled BSA (ATTO488-BSA, Jena Bioscience), β-galactosidase (*E. coli*, 540 kDa, Calbiochem, Darmstadt, Germany).

### CULTIVATION OF *LEISHMANIA TARENTOLAE* PROMASTIGOTES

*Leishmania tarantolae* promastigote cells (LEXSY host P10) were obtained from Jena Bioscience and cultivated under the recommended conditions, as described previously [Breitling et al., 2002; Kushnir et al., 2005]. They were grown as suspension

TABLE I. Used Cell Penetrating Peptides

| | | |
|---|---|---|
| Amphiphilic-cationic peptides (transcription factors and -activators) | | |
| Penetratin (Antennapedia) | RQIKIWFQNRRMKWKK | 8 positive charges, MW 2,247 Da |
| HIV-TAT (47–57) | YGRKKRRQRRR-amide | 9 positive charges, MW 1560 Da |
| MPG-peptides | | |
| MPGα | Ac-GALFLAFLAAALSLMGLWSQPKKKRKV-NH-$CH_2$-$CH_2$-SH | 5 positive charges, MW 3,047 Da |
| MPGβ | Ac-GALFLGFLGAAGSTMGAWSQPKKKRKV-NH-$CH_2$-$CH_2$-SH | 5 positive charges, MW 2,910 Da |
| CAD-2 (des-Acetyl, $Lys^{19}$-CADY) | GLWRALWRLLRSLWRLLWKA-NH-$CH_2$-$CH_2$-SH | 6 positive charges, MW 2,653 Da |
| Cell penetrating pentapeptide | | |
| CPPP-2 | KLPVM | 2 positive charges, MW 605 Da |
| Histone | | |
| Calf thymus histone, type II-AS | More than 100 amino acids | Many positive charges, resulting from Arg-, Lys-residues (more than 20) |

culture at 26°C in complex medium LEXSY BHI (Jena Bioscience) completed with Hemin (final concentration of 5 μg/ml, Jena Bioscience) and 1% penicillin/streptomycin (Jena Bioscience). Ten milliliter of stock cultures with an optimum OD of 1.4 to 1.8 (ca. 5–8 × $10^7$ cells/ml) were diluted twice per week at 1:20 or 1:50 ratios.

### PREPARATION OF CPP STOCK SOLUTIONS

CPPs (0.5 mg) were dissolved in 1.25 to 1.50 ml of sterile and oxygen free water. To obtain a ten-time higher molar ratio with CPPP-2, 1.2 mg was dissolved in 1 ml oxygen-free water according to the inventors [Gomez et al., 2007; Gomez and Matsuyama, 2011]. The solution was thoroughly mixed, frozen to −80°C and thawed (repeated three times), and sonicated for 5 min. The resulting stock solutions were used immediately or stored as aliquots at −20°C.

### FORMATION OF THE COMPLEXES WITH PROTEINS

A detailed description of the formation of complexes with peptides and proteins is given in [Mussbach et al., 2011b]. Briefly, cargo proteins (ATTO488-BSA, MW ≈ 68 kDa; β-gactosidase, MW ≈ 540 kDa) and the calculated volume of stock solution of CPPs were dis-solved separately in 100 μl of phosphate buffer solution (Dulbecco′s phosphate buffer solution 1x (PBS), pH 7.0 to 7.5; PAA, Pasching Austria). Both solutions were thoroughly mixed by repeated pipetting. The mixture was incubated for 30 min at 37°C to achieve complex formation. The molar ratio of cargo to CPPs was calculated to 1:10. For internalization of higher amounts both the amount of protein and stock solution of CPP were multiplied.

### TRANSDUCTION OF LEISHMANIA TARENTOLAE

For transduction, cells in an early stage of development were used. Stock cultures were diluted 1:10 the day before the experiment in 10 ml of culture medium in 25 $cm^2$-ventilated tissue culture flasks and incubated overnight in the dark at 26°C for optimal proliferation. Cell suspensions with an OD of 1.2 (ca. 4 × $10^7$ cells/ml) were used for transduction. The cells were thoroughly washed twice with PBS (5 ml each) by centrifugation (2000 × g, 3 min, "Eppendorf 5424", Eppendorf, Hamburg, Germany). Pellets were resuspended in the original volume of PBS containing 0.1% BSA (Jena Bioscience) and aliquoted into 24-well plates. 400 μl of cell suspension were used per experiment. 200 μl of the complex solutions were added to these cells, and mixtures were incubated for 1 h at 26°C in the dark with gentle agitation. Successively, 1 ml of complete growth medium (LEXSY BHI) was added per sample and incubation was continued at 26°C for further 2 h. Cells were washed twice with PBS (2 ml each) by centrifugation (2000 × g, 3 min) prior to analysis of transduction by formation of a stained β-galactosidase product or monitoring the fluorescence by ATTO488-BSA.

### DEGRADATION OF PENETRATIN BY PROTEASES

*Leishmania tarentolae* cells were cultivated overnight under optimized conditions. Then the complete medium was removed by centrifugation and the washed cells ($10^8$) were incubated with penetratin in PBS. Incubation was performed under the same conditions as the transduction experiments with PBS for 1 h at 26°C. All substrates (*para*-nitroanelids from succinyl-Phe, succinyl-L-Arg, succinyl-Ala-Ala-Ala, and succinyl-Ala-Ala-Pro-Phe) used for detecting the type of protease activity were purchased from Sigma-Aldrich. Penetratin was incubated with cell-free culture filtrate (from 4 × $10^7$ cells) and with centrifuged homogenate (from 1 × $10^8$ cells). After 15, 30, 45 and 60 min samples of 200 μl were taken and the proteolytic reaction was stopped by addition of 50 μl 37% HCl. After freezing and lyophilization the samples were analyzed by analytical high performance liquid chromatography (HPLC, Shimadzu LC-10AT chromatograph for penetratin, and JASCO PU-987, JASCO, Gotha, Germany for other CPPs) using an RP18 column (Vydac, 218TP). The gradient used was: 10% B to 50% B in 40 min; A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile. The flow rate was 1.0 ml/min and detection was accomplished at 220 nm.

### STAINING OF INTERNALIZED β-GALACTOSIDASE

After finishing the transduction protocol described above, staining of *Leishmania* cells was performed in multiwell-plates using a staining solution containing potassium ferrocyanide, potassium ferricyanide and X-Gal (99%, Calbiochem, Schwalbach, Germany). Cells were incubated in staining solution overnight and directly observed in a microscope (Nikon Eclipse TS100, Nikon, Düsseldorf, Germany) using a 400-fold magnification. The values given in Table II are calculated from the density of the colored product formed by the internalized active enzyme β-galactosidase. The image of the stained 24-well plate was converted into grey values using software Adobe Photoshop. In regions of interest (ROIs) with identical sizes to each well, including the background well, the mean density was measured. Using Microsoft Excel the measured density of background well was subtracted. Listed intensities in Table II are directly proportional to the density of the corresponding well.

### QUANTITATIVE ANALYSIS OF UPTAKE OF ATTO488-BSA INTO *LEISHMANIA TARENTOLAE* CELLS AND CALCULATION OF INTRACELLULAR AMOUNTS AND CONCENTRATIONS

*Leishmania* cells were transduced with ATTO488-BSA under standard conditions (4 × $10^6$ cells per well, 1 h at 26°C, PBS-buffer solution, containing 0.1% BSA) and incubated for 2 h with complete BHI medium at 26°C. After finishing transduction cells were thoroughly washed three times with PBS (2 ml each) by spinning down for 3 min at 2000 × g. For achieving a homogenous distribution of ATTO-BSA for fluorescence measurements the cells were lysed with 900 μl of lysis

TABLE II. Effectivity of Internalization of β-Galactosidase into *Leishmania Tarentolae* Cells by Used Cell Penetrating Peptides

| | Intensity of colored product formed by internalized active β-galactosidase | | |
|---|---|---|---|
| | Molar ratio of non-covalent complexes (CPP: β-Gal) | | |
| Transporter | 5:1 | 10:1 | 20:1 |
| Penetratin (Antennapedia) | 0 | 0 | 0 |
| HIV-TAT (47–57) | 0 | 0 | 0 |
| MPGα | 30 | 50 | 80 |
| MPGβ | 2 | 20 | 25 |
| CAD-2 (des-Acetyl, $Lys^{19}$-CADY) | 70 | 105 | 105 |
| CPPP-2 | 0 | 0 | 3 |
| Calf thymus histone, type II-AS | 10 | 10 | 40 |

reagent, formed from PBS by the addition of Triton X-100 (Eurosio, Cortaboeuf, Frankreich) to reach a concentration of 1%. Resulting homogenous solutions were transferred by 100 µl aliquots to 96-well-plates for quantitative fluorescence detection (excitation at 485 nm; emission at 520 nm). The fluorescence measurements were performed with a plate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany). Each CPP and control concentration was measured in six separate wells. Fluorescence values used for further calculations were taken from mean values reduced by mean values of control. Control values were measured by addition of ATTO488-BSA in corresponding concentrations to the transduction medium, but without CPPs. Fluorescence intensities were compared to the standard (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 µg ATTO488-BSA, molecular weight $68 \times 10^3$ Da). By the sixfold measurements the standard deviations of fluorescence values were in the range of about five percent from the value.

Division of the value calculated for the internalized amount of fluorescent protein ATTO488-BSA by the cell number ($4 \times 10^6$) gives the internalized amount for one cell. The mean volume of *L. tarentolae promastigote* cells was calculated as $2.0 \times 10^{-7}$ µL. The amount of internalized fluorescent cargo divided by the mean cell volume provides the intracellular concentration.

### CONFOCAL FLUORESCENCE MICROSCOPY

For confocal microscopy of fixed and living cells, a LSM 710 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) was used as previously described [Brand et al., 2010]. Briefly, samples were scanned using a 63x Plan-Apochromat oil immersion objective. DAPI (Sigma-Aldrich Chemie, Taufkirchen, Germany) and ATTO488 fluorescent dyes were excited by laser light at 405 nm and 488 nm, respectively, and their emissions were recorded using fluorophore-specific band path settings (430–480 nm and 505–530 nm, respectively). To avoid bleed-through effects, each dye was scanned independently in a multitracking mode. Differential interference contrast (DIC) images were acquired with the 488 nm laser line along with scanning of the ATTO488 signals. DAPI was used for staining of double-stranded DNA. Note that in Figure 3 the DAPI signal is false-colored in red. Cells were transduced for the above described procedure with 10 µg ATTO488-BSA using a tenfold molar excess of CPPs. Transduction and measurement were performed under strictly the same conditions for all used CPPs allowing comparison each other.

### CYTOTOXICITY TEST FOR *LEISHMANIA TARENTOLAE*

*Leishmania* cells were cultivated as described previously, resuspended in PBS and treated with increasing concentrations of CPPs for 1 h. Cytotoxic effects were monitored by changes in morphology and motility which were observed by using a microscope at 400-fold magnification (Nikon Eclipse TS100, Nikon, Düsseldorf, Germany).

## RESULTS AND DISCUSSION

### SELECTION OF CELL-PENETRATING PEPTIDES FOR FORMATION OF NON-COVALENT COMPLEXES WITH BOTH PROTEINS

We used CPPs that form non-covalent complexes to allow for easier handling, and the intracellular auto-release of cargoes. In our previous studies [Mussbach et al., 2011a,b; Keller et al., 2013] we used the cell penetrators listed in Table I. They belong to different types, derived from transcription factor (penetratin) [Dupont et al., 2011], transcription activator (HIV-TAT) [Ignatovich et al., 2003; Chen et al., 2007; Dai et al., 2011], from BAX-inhibitory peptide (CPPP-2) [Gomez et al., 2007; Gomez and Matsuyama, 2011], and histones [Hariton-Gazal et al., 2003], or were designed by combining partial sequences from different functional peptides (MPG-peptides) [Deshayes et al., 2004, 2008; Crombez et al., 2009; Kurzawa et al., 2010]. All peptides used in this study contain a relatively high number of positive charges. Distribution of positive charges and hydrophobic side chains differ in the amino acid sequences. Except for CAD-2 and CPPP-2 all other peptides contain nuclear localization sequences. Since for most peptides a tenfold excess of CPP over cargo-protein is recommended, the pentapeptide CPPP-2 requires a 100-fold excess. In our studies on mammalian cells [Mussbach et al., 2011a], penetratin and HIV-TAT showed only very low transport efficiency for proteins. Only penetratin was able to transport nucleotides as non-covalent complexes into mammalian cells. To find species-dependent differences regarding internalization we also used both penetratin and HIV-TAT in this study.

### PROTEOLYTIC ACTIVITY OF *LEISHMANIA TARENTOLAE*

Cells are able to degrade peptides and proteins by proteolytic cleavage. To check the proteolytic activity of *L. tarentolae* we used different substrates and studied the degradation of penetratin. Starting with completely unspecific azocasein we used more specific substrates to differentiate between types of protease activities. Using *para*-nitroanelids with the following sequences succinyl-Phe (chymotrypsin), L-Arg (trypsin), succinyl-Ala-Ala-Ala (elastase) and succinyl-Ala-Ala-Pro-Phe (elastase and chymotrypsin) we found that secreted (culture filtrate), as well as membrane-bound (washed intact cells) and cytosolic proteases (homogenate) have mainly chymotrypsin-like activity. These results correspond with the well-described surface-bound and secreted $Zn^{2+}$-dependent metallo-protease Leishmanolysin (EC 3.4.24.36, gp63) [McGwire et al., 1996; Jaffe and Dwyer, 2003]. This surface-protease can be extracellularly released from its glycosylphosphatidyl- (GPI-) anchor [McGwire et al., 2002], splits the peptide bond of tyrosine [Corradin et al., 1999], but seems to be less active in the *L. tarentolae* strain [Raymond et al., 2012].

While the internalization process proceeds at normal temperatures for 20 to 120 min (as commonly accepted) we studied the proteolytic degradation of the CPP penetratin during this time and under conditions used for the internalization of cargoes ($4 \times 10^7$ cells). For monitoring, the cleavage samples were taken after 15, 30, and 60 min, stopped by the addition of conc. HCl, lyophilized and analyzed by HPLC. Figure 1 shows that within 60 min the penetratin has been fully degraded by the washed intact cells, culture filtrate, and also by cytosolic proteases from the homogenate. Similar studies on mammalian cells have shown that these cells are also able to degrade penetratin and other CPPs. However, the CPPs are differently stable against proteolytic cleavage. In mammalian cells the most stable CPP is CAD-2 whereas penetratin is the most unstable [Keller et al., 2013]. Despite cleavage by proteases the CPPs are able to transport cargoes into both mammalian and *Leishmania* cells. The CPPs were used in a 10-fold molar excess to form non-covalent complexes with the cargo

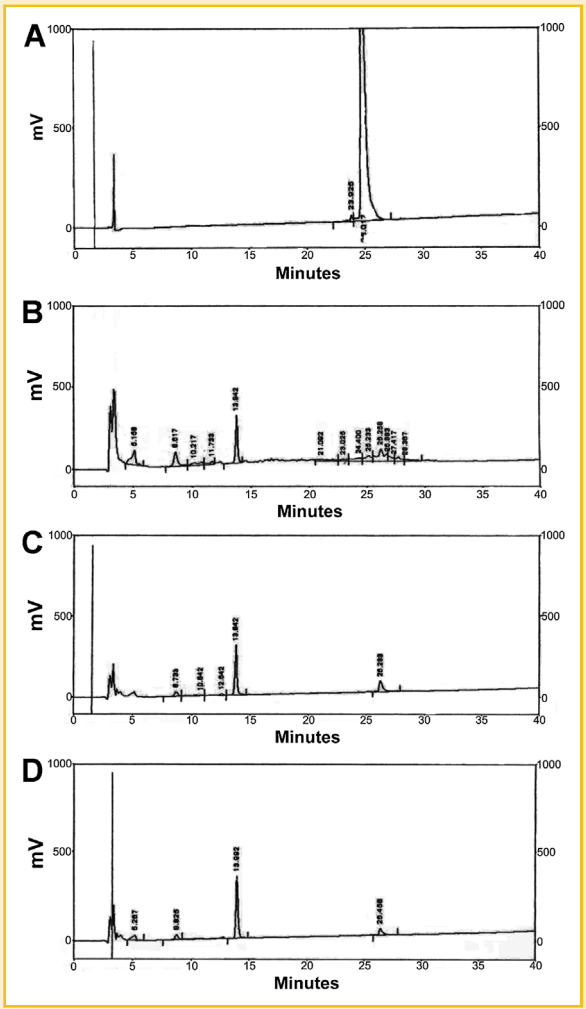


Fig. 1. Degradation of penetratin by proteases from *Leishmania tarentolae*. Penetratin was incubated without (A) or with washed cells (B), culture filtrate (C), or cell homogenate (D). In the latter three cases the peptide was degraded in less than 60 min, the HPLC-peak of penetratin at 25 min, as shown in (A), completely disappeared.

and the uptake process required less than 60 min. We suggest that the proteolytic stability of a CPP contributes to its uptake efficiency. For therapeutic purposes the proteolytic degradation of CPPs is vice versa an advantage, leading to an intracellular auto-release of cargo and providing non-toxic amino acids from the CPPs.

## INTERNALIZATION OF β-GALACTOSIDASE

The large enzyme β-galactosidase with a molecular weight of 540 kDa is composed of four equal subunits. It forms complexes with 5, 10, or 20 molecules of the corresponding relatively small CPP molecules. The protein-CPP complexes are stabilized by non-covalent interactions at the protein surface. The driving force behind these processes is the formation of ionic, hydrophobic, dipole–dipole, and cation–π-electron interactions. The formation of stable, non-covalent complexes also requires, besides suitable conformational shapes of the surface regions, conformational flexibility of both binding partners.

Internalization is a very complex process comprised of a multitude of different steps. These include interaction with proteoglycans at the cell surface [Guha-Niyogi et al., 2001; Ziegler and Seelig, 2008], with lipids in the micro-heterogeneous areas of the cell membrane, scavenger receptors [Lindberg et al., 2013], or syndecans [Letoha et al., 2010], uptake into the cytosol using contractile proteins, in certain cases formation, processing, and release from endosomes and finally intracellular trafficking. Due to the considerable complexity of these numerous processes, we focused only on the most important parameter, the transported cargo. The internalized active enzyme forms a colored product from the substrate X-Gal which is easily detectable. Because *Leishmania* are suspension cells we performed the staining reaction in the whole well and compared the resulting colors semi-quantitatively. Table II indicates that increasing molar ratios between enzyme and CPPs increases the uptake. CAD-2 has the highest uptake efficiency, followed by MPGα and histone. The pentapeptide CPPP-2 has only a very small effect. Although both designed peptides, MPGα and MPGβ, respectively, have very similar amino acid sequences they transport the enzyme with strongly different efficiencies. Despite the distinct differences between mammalian and *Leishmania* cells in the composition of surface-glycoprotein, lipid composition of the membrane, signal pathways, and intracellular processes, the enzyme was transported with a similar rank order of internalization efficiencies for the used CPPs.

HeLa and Jurkat cells: MPG≥MPGβ>CAD-2»CPPP-2//HIV-TAT, penetratin [Mussbach et al., 2011a; Keller et al., 2013]
Leishmania cells: CAD-2>MPGα>Histone>MPGβ»CPPP-2//HIV-TAT, penetratin

This is an interesting result. It shows that for the internalization process the properties of the complex seem to be more important than the cells properties. Furthermore we found in good agreement with our previous studies with mammalian cells, that HIV-TAT and also penetratin were unable to transport proteins into *Leishmania* cells.

## INTERNALIZATION OF ATTO488-BSA

The cargo ATTO488-BSA is synthesized by labeling bovine serum albumin with a fivefold molar excess of N-hydroxy-succinimid ester of the fluorescent label ATTO488 and contains on average five fluorescent labels per one BSA molecule. The internalized fluorescent cargo was quantitatively measured after lysis of the cells. Control values were obtained by lysis of blank cells or by lysis of cells incubated with corresponding concentrations of ATTO488-BSA but without CPPs. Controls showed only very low fluorescence values. This finding indicates low uptake and weak adsorption of the fluorescent cargo itself. The high ability of BSA for general binding of peptides, proteins, and other compounds on its surface is well-known and is possibly enhanced by the hydrophobic fluorescent label. As seen in Figure 2 this cargo-protein was internalized into *Leishmania* by MPGα with the highest efficiency. CAD-2 was less active. The following rank orders for internalization of ATTO488-BSA into HeLa and Leishmania cells were estimated:

HeLa cells: MPGα>MPGβ>CAD-2»CPPP-2//HIV-TAT, penetratin [Mussbach et al., 2011a; Keller et al., 2013]
Leishmania cells: MPGα>histone>MPGβ»CAD-2>CPPP-2//HIV-TAT, penetratin

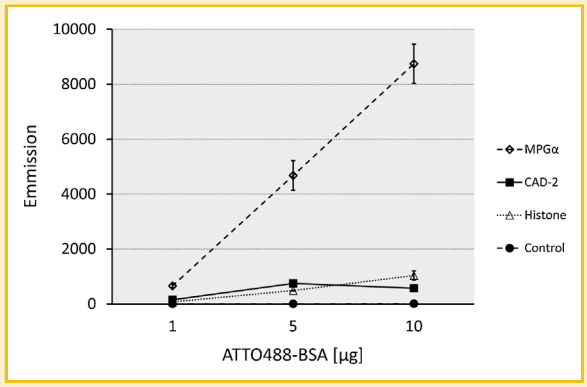


**Fig. 2.** Quantification of ATTO488-BSA uptake into *Leishmania* promastigote cells. Fluorescent-labeled BSA was internalized into *Leishmania* cells via formation of non-covalent complexes with CPPs in a ratio of 1:10. After cell-lysis the fluorescence of the whole well was measured. The figure indicates that increasing concentrations of the complex with MPGα linearly increased the cargo uptake. Complexes with other CPPs were less efficient. Quantification of fluorescence was used to calculate intracellular amounts and concentrations.

How can the differences in the uptake efficiencies between the CPPs and the two different cargo-proteins be explained? The complex process of cargo internalization begins with the formation of non-covalent complexes; the lacking transport activity of penetratin and HIV-TAT for both proteins seems to result from a weak complex formation. Because of their numerous positive charges both transporting peptides are much more polar than other CPPs used in this study. The designed peptides MPGα, MPGβ, and CAD-2 contain, in addition to the positively charged residues, many hydrophobic side chains of amino acids. This finding seems to indicate that hydrophobic interactions between CPPs and the surface of the proteins used here are more important in the formation of stable, non-covalent complexes than the ionic interactions.

To explain exactly these differences in complex formation and internalization of both cargo-proteins β-galactosidase and ATTO488-BSA, respectively, more detailed knowledge is required about the protein surfaces and conformation and conformational flexibility of the applied CPPs. Yet, we can only assume that CAD-2 fits better to the surface of the large enzyme β-galactosidase than MPGα and conversely for ATTO488-BSA.

### INTRACELLULAR DISTRIBUTION

In mammalian cells, such as HeLa, the internalized cargo ATTO488-BSA was found in the cytosol, vesicles and in the nucleus [Mussbach et al., 2011a]. Surprisingly, in *L. tarentolae* fluorescent cargo was found only in the kinetoplast and the nucleus. This finding was confirmed by co-staining of double-stranded DNA with DAPI, which demonstrated the same intracellular distribution. Only the kinetoplast and nucleus were stained. Co-localization of ATTO488-BSA and DAPI staining indicates specific and effective transport of the cargo into both organelles. No fluorescence was found in the cytosol or vesicles. Figure 3 shows that other CPPs were internalized with lower efficiency, but in nearly the same efficiency as estimated in Figure 2 by quantification of fluorescence for the whole well containing about $1.6 \times 10^7$ cells. Although all confocal microscope fluorescence studies were performed under strictly identical conditions the figures show only a snapshot with very few cells. Thus quantitative and semiquantitative comparisons between both methods are only to some degree possibly. Since MPGα contains a nuclear localization sequence the transport into the nucleus can be expected. Yet, in higher concentrations ATTO488-BSA alone, in the absence of any CPPs, can also be detected to a lower extent in the nucleus. This finding corresponds with our fluorescent microscopic studies performed on HeLa cells [Mussbach et al., 2011a]. As the fluorescent cargo was mainly found in the nucleus and kinetoplast, we did not use endosomolytic auxiliaries such as chloroquine, wortmannin or polyethylene imines in the present study.

### INTRACELLULAR AMOUNTS AND CONCENTRATIONS

The internalized amount of ATTO488-BSA fluorescence was quantified, the cell number per well estimated and the mean cell volume calculated from microscopic measurements. Division of internalized amount of cargo by the cell number gives the intracellular amount of cargo; this value divided by cell volume reveals the intracellular concentration of cargo. The transported amount of ATTO488-BSA is linearly dependent on the amount of complex formed with MPGα. The highest number of fluorescent BSA molecules achieved in one *Leishmania* cell was approximately $1.2 \times 10^5$. Table III shows that, despite the lower cell volume of *Leishmania*, the intracellular concentrations reached similar levels as in HeLa cells. Because the amount per cell corresponds to the cell volume, these values are about 100-fold lower than for HeLa cells. Based on quantitative fluorescence measurements with other CPPs, we also calculated internalized amounts and intracellular concentrations for these CPPs. Despite the strongly lower transport efficiencies, histone and CAD-2 also internalize about $10^3$ cargo molecules into a single *Leishmania* cell.

### AUXILIARIES

Only BSA was used to protect CPPs and cargoes from proteolytic degradation, and to enhance uptake by acting as a nutrient. BSA acts without any toxicity.

### CYTOTOXICITY

Most CPPs have a low cytotoxicity to *L. tarentolae* (listed in Table IV). Thus penetratin, HIV-TAT, CAD-2, histone [Singh et al., 2010], and CPPP-2 were tolerated in concentrations up to 100 µg/ml. *Leishmania* were more susceptible to both amphipathic peptides MPGα and MPGβ, respectively, than the mammalian cells. We believe that both compounds act on *Leishmania* as antimicrobial peptides.

## CONCLUSIONS

*Leishmania tarentolae* can be transduced with proteins by formation of non-covalent complexes with suitable CPPs. Our investigation highlights an important role of hydrophobic interactions in the formation of protein-CPP complexes.

Surface-bound and secreted proteases, mainly with chymotrypsin-like activity, are involved in degradation of CPPs and cargoes. CPPs

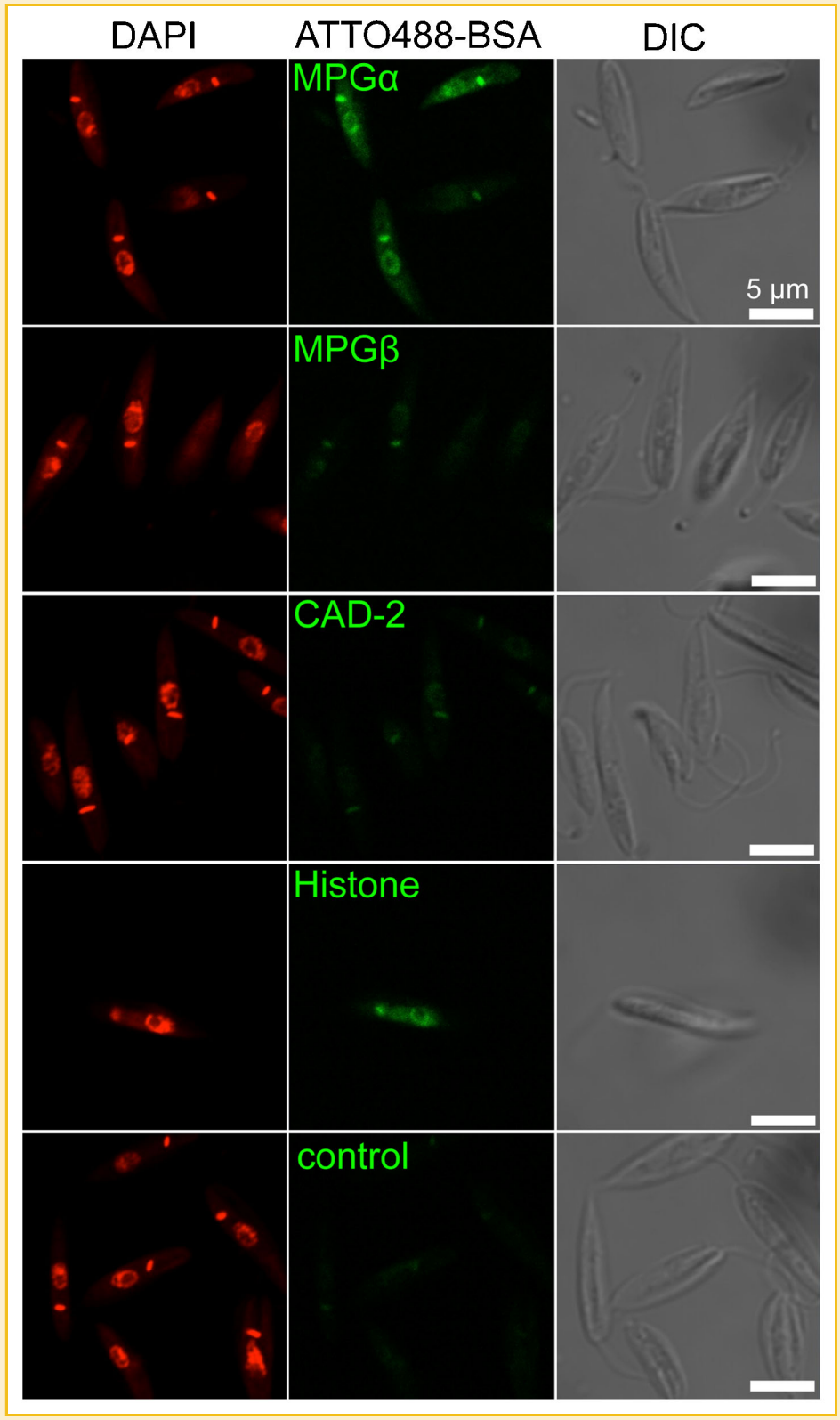


**Fig. 3.** Intracellular localization (distribution) of ATTO488-BSA in *Leishmania tarentolae*. The intracellular distribution of fluorescence revealed localization in nucleus and kinetoplast by using laser scanning confocal fluorescence microscopy. Co-staining with DAPI, a dye for double-stranded DNA, demonstrated co-localization of ATTO488 and DAPI dye fluorescence. Only a very slight fluorescence was found in the cytosol. No vesicular staining was observed.

TABLE III. Internalized Amounts of Fluorescent Cargoes and Reached Intracellular Concentrations in *Leishmania Tarentolae* and HeLa Cells

| Amount of added complex per 0.6 ml PBS (1 h) + 1 ml BHI-medium (2 h) | Internalized amount and concentration ($4 \times 10^6$ *Leishmania tarentolae*-cells per well) | |
|---|---|---|
| | Amol per cell | Intracellular concentration (μM) |
| **Internalization of ATTO488-Bovine Serum Albumin (BSA) into *Leishmania tarentolae*** | | |
| MPGα | | |
| 1 μg ATTO488-BSA + MPGα, molar ratio 1:10 | $1.6 \times 10^{-2}$ | 0.008 |
| 5 μg ATTO488-BSA + MPGα, molar ratio 1:10 | $12 \times 10^{-2}$ | 0.6 |
| 10 μg ATTO488-BSA + MPGα, molar ratio 1:10 | $23 \times 10^{-2}$ | 1.2 |
| CAD-2 | | |
| 1 μg ATTO488-BSA + CAD-2, molar ratio 1:10 | $0.36 \times 10^{-2}$ | 0.002 |
| 5 μg ATTO488-BSA + CAD-2, molar ratio 1:10 | $2.6 \times 10^{-2}$ | 0.01 |
| 10 μg ATTO488-BSA + CAD-2, molar ratio 1:10 | $2.0 \times 10^{-2}$ | 0.01 |
| Histone | | |
| 1 μg ATTO488-BSA + Histone, molar ratio 1:10 | $0.27 \times 10^{-2}$ | 0.001 |
| 5 μg ATTO488-BSA + Histone, molar ratio 1:10 | $1.62 \times 10^{-2}$ | 0.008 |
| 10 μg ATTO488-BSA + Histone, molar ratio 1:10 | $2.2 \times 10^{-2}$ | 0.01 |
| **Amount of added complex per 1.6 ml serum-free medium** | **Internalized amount ($0.3 \times 10^6$HeLa cells per well)** | |
| Comparison to HeLaCells | | |
| ATTO488-deoxy Uridine Triphosphate into HeLa cells [Mussbach et al., 2011b] | | |
| 1 μg + JBS-Nucleoducin, charge by charge 1:4 | 1.1 | 0.1 |
| ATTO488-bovine serum albumin into HeLa cells [Mussbach et al., 2011a] | | |
| 25 μg + JBS-Proteoducin, molar ratio 1:10 | 50 | 4.3 |
| FITC-Antibody (secondary) into HeLa cells [Mussbach et al., 2011a] | | |
| 25 μg + JBS-Proteoducin, molar ratio 1:10 | 4.3 | 0.6 |

are differently stable against this proteolytic degradation. From the CPPs used in this study, penetratin was the most unstable; the strongly hydrophobic CAD-2 was the most stable. To maintain proteolytic stability during transduction (requiring one or more hours), the most optimal CPP for a given task must be considered.

Both the enzyme β-galactosidase (MW = 540 kDa) and the fluorescent-labeled BSA (MW = 68 kDa) can be transported with similar high efficiency into *Leishmania*, despite their different molecular weights. However both cargo-proteins were internalized with strongly different rank orders by the CPPs. The differences between rank orders for both cargo proteins are larger than the differences for a given cargo between HeLa and *Leishmania* cells. This finding may indicate that formation of stable non-covalent complexes from cargo-protein and CPP is more important for internalization than differences in the surfaces of both cell types.

The intracellular concentration of ATTO488-BSA reaches the same low micromole range as in mammalian cells. The highest fluorescence was found in the nucleus and kinetoplast, which agrees well with the staining of double-stranded DNA using DAPI. In contrast, only very little or no fluorescence was detected in the membrane or the cytoplasm.

TABLE IV. Cytotoxicity of Cell Penetrating Peptides on *Leishmania Tarentolae* Cells

| | |
|---|---|
| Amphiphilic-Cationic Peptides transcription factors and -activators) | |
| Penetratin (Antennapedia) | <50 μg/ml |
| HIV-TAT (47–57) | ≥100 μg/ml |
| MPG-peptides | |
| MPGα | ≤30 g/ml |
| MPGβ | ≤30 μg/ml |
| CAD-2 | <100 μg/ml |
| Cell penetrating pentapeptide | |
| CPPP-2 | ≥100 μg/ml |
| Histone | |
| Calf thymus histone, type II-AS | >100 g/ml |

*Leishmania* are more sensitive to MPG-peptides than other investigated mammalian cells, indicating a kind of antimicrobial activity of these peptides.

In summary: Our transduction studies on *Leishmania* are necessary for developing protocols for internalization of cargoes into Leishmania and to estimate the cytotoxicity of favored CPPs. Thus these studies are important in demonstrating the ability to regulate protozoa cells by using activators or inhibitors to influence transcriptional and signal pathways.

## ACKNOWLEDGMENTS

We gratefully acknowledge the valuable studies on proteolytic stability of CPPs by our former research students Claudia Lueck, Nadja Fischer and Linn Stelter. We thank Dr. Regina Pietrucha for her contributions to the cytotoxicity studies.

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