ELSEVIER

Biochimica et Biophysica Acta 1564 (2002) 365–374

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

A quantitative validation of fluorophore-labelled cell-permeable peptide conjugates: fluorophore and cargo dependence of import

Rainer Fischer, Thomas Waizenegger, Karsten Köhler, Roland Brock*

Group of Genomics and Proteomics, Center for Bioinformatics Tübingen, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany

Received 27 February 2002; received in revised form 16 May 2002; accepted 4 June 2002

Abstract

Cell-permeable peptides were evaluated for a quantitatively controlled import of small molecules. The dependence of the import efficiency on the fluorophore, on the position of the fluorophore as well as on the nature of the cargo were addressed. Cellular uptake was quantitated by flow cytometry and fluorescence correlation microscopy (FCM). Fluorophores with different spectral characteristics, covering the whole visible spectral range, were selected in order to enable the simultaneous detection of several cell-permeable peptide constructs. The transcytosis sequences were based either on the sequence of the Antennapedia homeodomain protein (AntpHD)-derived penetratin peptide or the Kaposi fibroblast growth factor (FGF)-derived membrane translocating sequence (MTS)-peptide. In general, the AntpHD-derived peptides had a three- to fourfold higher import efficiency than the MTS-derived peptides. In spite of the very different physicochemical characteristics of the fluorophores, the import efficiencies for analogues labelled at different positions within the sequence of the import peptides showed a strong positive correlation. However, even for peptide cargos of very similar size, pronounced differences in import efficiency were observed. The use of cell-permeable peptide/cargo constructs for intracellular analyses of structure–function relationships therefore requires the determination of the intracellular concentrations for each construct individually.

Keywords: Cell-permeable peptide; Cellular uptake; Flow cytometry; Fluorescence; Fluorescence correlation microscopy; Target validation

1. Introduction

Cell-permeable peptides cross the plasma membrane in a receptor- and energy-independent manner [1,2]. These molecules were either derived from the hydrophobic sequences of signal peptides, such as the Kaposi fibroblast growth factor (FGF)-derived membrane-translocating sequence (MTS) [3], or from insect proteins, such as the third helix of the DNA-binding domain of the Antennapedia homeodomain protein (AntpHD) [2]. While the former is a highly hydrophobic sequence with 8 out of 16 amino acids being valine and leucine residues and the remainder either alanine or proline, the latter is basic with 7 out of 16 residues being arginine or lysine.

Covalent or noncovalent [4] linkage of cell-permeable peptides to other molecules mediates the noninvasive import of these cargo molecules into cells ex vivo, as well as in whole animals [5,6]. Such cargo have been peptides [7,8], oligonucleotides [4], as well as proteins as large as 120 kDa [6,9]. The capacity to mediate entry of impermeable molecules into cells renders cell-permeable peptides a valuable tool in the early stages of the drug development process. Import of inhibitors may serve to identify and validate intracellular molecules as targets for achieving a desired therapeutic outcome [10]. Substances exhibiting biological activity for isolated molecules in vitro, but unfavourable pharmacokinetic properties for in vivo tests, may be rendered cell-permeable. Fusion of cell-permeable peptides to antisense-oligonucleotides has been employed for suppressing the expression of certain proteins [11]. Peptide conjugates have served as pseudo-substrates, competitive inhibitors of the enzyme active site or structural mimics of the interaction domain [12]. The advantages of peptide-based approaches are the accessibility of large collections of different compounds by well-established automated procedures, as well as the generation of active compounds based on available

* Corresponding author. Tel.: +49-7071-2977629; fax: +49-7071-295359.
E-mail address: roland.brock@uni-tuebingen.de (R. Brock).

0005-2736/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(02)00471-6

structural information of interaction domains. Structure–function relationships of intracellular interaction domains have been analysed by testing a series of different peptides fused to a cell-permeable moiety [13].

If the import efficiency of cell-permeable constructs is cargo-dependent, the determination of the intracellular concentrations for each of the cell-permeable peptide/cargo constructs is mandatory, particularly in the analysis of structure–function relationships. Otherwise, differences in a biological response may simply reflect differences in uptake efficiency. Fluorescent derivatization of cell-permeable peptides provides a sensitive and specific quantitative read-out on intracellular peptide concentration using established techniques such as flow cytometry. In addition, the analysis of the subcellular distribution of cell-permeable peptide/cargo constructs by fluorescence microscopy enables the identification of cellular compartments targeted by a cell-permeable construct. Attaching the fluorescent reporter group to the cell-permeable moiety minimizes the risk of interference of the fluorescent label with the activity of the cargo. By means of chemical ligation [14] or disulfide linkage [15], cell-permeable peptides or fluorescent analogues of these peptides may be used as building blocks for rendering molecules cell-permeable.

To date, however, fluorescent labelling of cell-permeable peptides has been limited to N-terminal derivatization of peptides either with fluorescein as described for example in Refs. [16,17] or NBD (7-nitrobenz-2-oxo-1,3-diazol-4-yl) [18]. The import of cell-permeable constructs into cells expressing GFP fusion proteins, as well as the simultaneous detection of several different cell-permeable constructs, requires the identification of spectrally distinct fluorophores compatible with import. For this purpose, it needs to be determined to which degree reporter groups with different physicochemical characteristics influence the import characteristics of the cell-permeable constructs. A fluorescein reporter group, for example, carries negative charge, while a tetramethylrhodamine (TAMRA) reporter group is zwitterionic. Positive charge as well as hydrophobicity were identified as important physicochemical determinants for import efficiency for the AntpHD peptide [2,19]. It was therefore to be expected that the import efficiency of the transcytosis sequences should be affected by the physicochemical characteristics of the fluorophore. Furthermore, fluorescent analogues carrying the fluorophore at a residue within the peptide rather than at the N terminus are still missing. Such analogues would enable the N-terminal modification of cell-permeable peptides with cargo molecules by solid phase synthesis. In addition, the introduction of one fluorophore within the transcytosis peptide and a second reporter group within the cargo would enable the tuning of the spectral characteristics of double-labelled constructs for fluorescence resonance energy transfer (FRET) [20]. Such molecules are valuable tools for the intracellular monitoring of, e.g. kinase [21] or protease activity [22].

In order to establish fluorescently labelled cell-permeable peptides as pharmacokinetic modifiers for small molecules, the impact of a fluorescent tag on the ability of the cell-permeable peptide to cross the plasma membrane was addressed. For this purpose, sets of analogues labelled with different fluorophores were generated by solid-phase peptide synthesis. Fluorophores were attached to the side chain of lysine residues introduced at different positions within the peptide sequence, and the effect of different fluorophores on import was compared.

For both the AntpHD and MTS peptides, internally labelled analogues were identified with import efficiencies equivalent to the N-terminally labelled peptides. Surprisingly, the relative import efficiencies of peptides labelled either with fluorescein or TAMRA at the N terminus or within the peptide showed a strong positive correlation. This correlation of import efficiencies for fluorescein- and TAMRA-labelled cell-permeable peptide analogues demonstrates that the capacity for import resides within the structure of the peptide, and is little affected by the physicochemical characteristics of the fluorophore. This finding was confirmed by fluorescence correlation microscopy (FCM) [23] of fluorescein- and TAMRA-labelled peptides at lower nanomolar concentrations.

The spectral range of flourescent analogues was extended to the near infra-red by conjugation of an indocyanine dye with Cy5-like [24] spectral characteristics. Import of three different cell-permeable peptide analogues was simultaneously detected by confocal laser scanning microscopy of living cells.

Finally, the dependence of import on the cargo was addressed for small peptide cargos. Perez et al. [25] have presented one example, in which for one out of three structurally related constructs, import was completely abrogated. A thorough quantitative analysis of import efficiencies for small peptide cargos has been missing, however. For this purpose a panel of different cargo peptides of 8 to 10 amino acids in length, as well as peptides with free or amidated C termini were synthesized. The uptake ratios of these molecules differed by factors of up to 8. These findings underscore the need for an individual determination of intracellular concentrations in applications, such as the intracellular analysis of structure–function relationships and target validation. The results presented in this paper validate fluorescence as the analytical method of choice for these measurements.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized in a 15-μmol scale by solid-phase Fmoc-chemistry on an automated peptide synthesizer for multiple peptide synthesis (RSP5032, Tecan, Hombrechtlikon, Switzerland). Fmoc-protected amino acids were pur-

chased from Novabiochem (Heidelberg, Germany) and Fmoc-protected 8-amino-3,6-dioxaoctanoic acid (Ado) from Neosystem (Strassburg, France). Standard chemicals in peptide chemistry were obtained from Fluka (Deisenhofen, Germany) and Merck (Darmstadt, Germany), solvents were p.a. grade. The peptides were synthesized as free acids on a Cl−Trityl resin (capacity 0.5 mmol/g; Senn Chemicals, Dielsdorf, Switzerland) and as amides on Rink−amide resin (capacity 0.7 mmol/g; Senn Chemicals). The amino acid sequence of the AntpHD peptide was RQIKIWFQNRRMKWKK and of the MTS peptide AAVALLPAVLLALLAP. The MTS peptide was extended at its N and/or C termini with one Ado building block to increase its water solubility.

The resin-bound peptides were labelled at the N terminus in the presence of 5 equivalents of either 5(6)-carboxyfluorescein (Fluo; Fluka) or 5(6)-carboxytetramethylrhodamin (TAMRA; Fluka), and 5 equivalents of each N-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) in dimethylformamide (DMF) twice for 20 h.

N-terminal labelling of the resin-bound AntpHD-peptide with the free carboxylic acid of the S0387 fluorophore was carried out in the presence of 2 equivalents of S0387 (FEW chemicals, Wolfen), 5 equivalents HOBt and 5 equivalents DIC in DMF twice for 20 h.

Conjugation of fluorophores to the ε-amino group of lysine side chains was carried out via incorporation of Fmoc-Lys(Dde)-OH (Calbiochem-Novabiochem, Bad Soden, Germany) as a building block in peptide synthesis. After Fmoc-deprotection of the amino terminus of the resin-bound peptide with piperidine/DMF, the N-terminal amino group was blocked by acetylation (acetic anhydride/diisopropylethylamin/DMF (1:1:8), 2×30 min). Deprotection of the Dde protecting group was performed by treatment with 2% hydrazine monohydrate in DMF (v/v) twice for 3 min. The coupling of the fluorophore followed the same protocol as the one for N-terminal labelling.

The peptides were cleaved off the resin by treatment with 92.5% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), 2.5% ethanedithiole and 2.5% H_2O twice for 2 h each. The peptides were precipitated by addition of diethylether (−20 °C). Precipitated peptides were washed twice with cold ether. Finally, the peptides were dissolved in tertiary butanol (tBuOH)/H_2O, 4:1, and lyophilized.

The peptides were analysed by analytical reverse phase chromatography (RP)-HPLC using an H_2O (0.1% TFA)/acetonitrile (0.1% TFA) gradient on a Waters 600 System (Eschborn, Germany) with detection at 214 nm and by Maldi-TOF mass spectrometry (Hewlett-Packard G2025A). Peptides were purified by preparative RP-HPLC (Nucleosil 300 C18 column, 5 μm particle diameter, 250×20 mm; Grom, Herrenberg, Germany) on a Waters 600 Multisolvent Delivery System using optimized gradients.

2.2. Determination of concentrations of fluorescently labelled peptides by UV/VIS spectroscopy

Peptides were dissolved in dimethylsulfoxide (DMSO) to about 20 mg/ml. For the determination of the concentration, these stock solutions were diluted 1:10 in ddH_2O. This solution was further diluted 1:100 in 0.1 M Tris–HCl pH 8.8 and the absorbance measured. The concentrations of the stock solutions were calculated assuming $\varepsilon_{\text{carboxyfluorescein 492 nm}}$=75,000 l/(mol·cm). TAMRA-labelled peptides dissolved in DMSO to about 20 mg/ml were diluted 1:10 in ddH_2O followed by a 1:100 dilution in methanol. The concentrations of the methanol solutions were calculated assuming $\varepsilon_{\text{TAMRA 540 nm}}$=95,000 l/(mol·cm). The concentration of the S0387-labelled peptide was adjusted by measurement of the concentration by FCM.

2.3. Cell culture

The adherent, melanoma-derived SKMel 37 cell line was grown in a 5% CO_2 humidified atmosphere at 37 °C in Dulbecco's Modified Eagle's medium (DMEM) with GLUTAMAX I, 25 mM HEPES, 4500 mg/l D-glucose, pH 7.2 (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (PAN Biotech, Aidenbach, Germany), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Boehringer, Mannheim, Germany). Cells were passaged by trypsinization with trypsin/EDTA 0.05/0.02% (w/v) in phosphate-buffered saline (PBS; Biochrom, Berlin, Germany) every third to fourth day. For growth in chambered coverglasses (Nunc, Wiesbaden, Germany), cells were seeded at a density of 25,000/cm^2 and used for FCM measurements at a confluency of approximately 75%.

2.4. Flow cytometry

SKMel 37 cells were seeded at a density of 80,000 per well in 24-well plates (Sarstedt, Nümbrecht) in 400-μl serum-containing medium. One day later, the cells were washed with serum-free medium and incubated in 200-μl serum-free medium. After 2 h, peptides were added in concentrations as indicated in Section 3. Each condition was tested in duplicate. After a 2-h incubation, cells were washed with PBS and trypsinized. The cells were suspended in PBS containing 0.1% (w/v) BSA and 5 mM glucose. Samples were kept on ice and measured immediately. The fluorescence of 5000 vital cells was acquired. Vital cells were gated based on sideward scatter and forward scatter. For the determination of nonspecific uptake of fluorescein-labelled peptides, the peptide Fluo-Ahx-SFHTMSAAKLI-NH2 (Ahx, 6-aminohexanoic acid) was added in the same concentration as were the MTS or AntpHD analogues. This peptide was previously designed as a peptide ligand for the HLA-DRB1*1501 MHC class II molecule [26]. It was selected because of the different

physicochemical side-chain characteristics and its very different biological relevance.

2.5. FCM

FCM measurements were carried out on a ConfoCor2 fluorescence correlation microscope (Carl Zeiss, Jena), equipped with a SensiCam cooled 12-bit CCD camera (PCO Computer Optics, Kelheim, Germany). For image acquisition by whole-field epifluorescence microscopy, a filter set consisting of an HQ470/40 excitation filter, a Q495LP beam splitter, and an HQ525/50 detection filter (AHF Analysentechnik, Tübingen, Germany) was used for imaging of fluorescein. A combination of an HQ548/10 excitation filter, a Q565LP beamsplitter, and an HQ610/75 detection filter were employed for imaging of TAMRA-labelled samples. For FCM measurements of fluorescein-labelled peptides, the 488-nm line of an argon ion laser was reflected into the sample via an HFT488 beam-splitter and fluorescence detected with a 500–550-nm band-pass filter. Fluorescence of TAMRA-labelled peptides was excited with a 543 HeNe-laser and detected with an HFT 488/543 beam-splitter in combination with a BP 560–615 detection filter. For positioning along the optical axis, the z-scan option of the ConfoCor2 software was employed. Profiles of fluorescence along the optical axis were acquired at 0.4 kW/cm^2 (for both laser lines) at a resolution of 0.5 μm per step. Z-scans were saved by screen capture from the computer screen. The position of the detection volume in the *x*- and *y*-dimensions was determined by bleaching a film of fluorescein adsorbed to the surface of a microscope slide and determining the position of the bleached spot in an epifluorescence image. Positioning in *x* and *y* was based on real-time transmission images acquired by the CCD camera using a motorized stage. A series of five subsequent autocorrelation measurements was carried out over 30 s each, for every data point. Laser intensities were 40 kW/cm^2 for fluorescein and 16 kW/cm^2 for TAMRA. The individual autocorrelation functions were fitted one by one and those unaffected by initial photobleaching included for averaging.

Reference measurements were carried out for carboxyfluorescein, TAMRA, Fluo-AntpHD, Fluo-MTS, and the corresponding TAMRA-labelled peptides at concentrations of 25 and 75 nM in Hepes-buffered saline (HBS; 135 mM NaCl, 10 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Na–Hepes, pH 7.4), supplemented with 0.1% BSA and 5 mM glucose in chambered coverglasses without cells in a volume of 400 μl.

For the determination of import efficiencies, the cells were incubated with the fluorescently-labelled peptide in HBS, 0.1% BSA, 5 mM glucose in a volume of 400 μl. The respective concentrations were as indicated in the figures. After 1 h of incubation at 37 °C, FCM measurements were started at room temperature. Autocorrelation functions were recorded in the nucleus and in the buffer next to each cell. A minimum of eight cells was analysed for each condition. Autocorrelation functions were fitted with the built-in routines of the ConfoCor2 software.

2.6. Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Göttingen, Germany) equipped with a Plan-Apochromat 63×1.4 N.A. lens. Triple detection of fluorescein, TAMRA, and S0387-labelled peptides was performed using a filter set consisting of an HFT UV/488/543/633 beam splitter in combination with an NFT 545 beam splitter and a BP 505–530 band pass filter for fluorescein detection, an NFT 635 VIS beam splitter and a BP 560–615 detection filter for TAMRA detection, and an LP650 long pass filter for S0387-detection. To avoid cross-talk detection, the multi-track modality of the LSM was employed for image acquisition. Live cells were measured in chambered coverslips as in FCM measurements.

3. Results

Fluorescently labelled cell-permeable peptides were established as pharmacokinetic modifiers in intracellular target validation. For this purpose, the import capacity was compared for cell-permeable peptide analogues labelled with fluorophores at different positions along the peptide. Moreover, the dependence of import on the peptide cargo was determined. All peptides were generated by solid phase peptide synthesis and purified by preparative HPLC. Import of peptides into the human melanoma cell line SKMel 37 was quantitated in living cells after trypsinization by flow cytometry. FCM, as well as confocal laser scanning microscopy, were employed to fully interpret the results obtained by flow cytometry.

3.1. Internally labelled fluorescent analogues

To this end, import of cell-permeable peptides had only been observed for peptides labelled with fluorophores at their N terminus. In order to extend the range of fluorescently labelled cell-permeable peptides, analogues derivatized with a fluorophore within the peptide sequence were generated. The availability of such compounds would (i) significantly increase the options for the generation of doubly labelled cell-permeable peptide constructs and (ii) enable the N-terminal extension of fluorescently labelled cell-permeable peptides with cargo. Analogues were generated for both the AntpHD and MTS peptides. The uptake efficiencies were compared and the effect of different fluorophores on translocation properties addressed.

For both transcytosis peptides, series of analogues were synthesized in which one out of four consecutive residues in the central portion of the peptide was replaced by a lysine residue derivatized with carboxyfluorescein via its ε-amino

Table 1
Name and sequence of carboxyfluorescein-labelled AntpHD and MTS analogues

Name	Sequence
AntpHD WT(Fluo)*	Fluo-RQIKIWFQNRRMKWKK
AntpHD W6K(Fluo)	Ac-RQIKIK(Fluo)FQNRRMKWKK
AntpHD F7K(Fluo)*	Ac-RQIKIWK(Fluo)QNRRMKWKK
AntpHD Q8K(Fluo)	Ac-RQIKIWFK(Fluo)QNRRMKWKK
AntpHD N9K(Fluo)*	Ac-RQIKIWFQK(Fluo)NRRMKWKK
MTS WT(Fluo)*	Fluo-AAVALLPAVLLALLAP-Ado-A
MTS V9K(Fluo)	Ac-Ado-AAVALLPAK(Fluo)LLALLAP-Ado-A
MTS L10K(Fluo)*	Ac-Ado-AAVALLPAVK(Fluo)LALLAP-Ado-A
MTS L11K(Fluo)	Ac-Ado-AAVALLPAVLK(Fluo)ALLAP-Ado-A
MTS A12K(Fluo)*	Ac-Ado-AAVALLPAVLLK(Fluo)LLAP-Ado-A

Those peptides marked with an asterisk were also synthesized as carboxytetramethylrhodamine-labelled analogues. 'Ac' denotes an N-terminal acetylation, Ado represents an 8-amino-3,6-dioxaoctanoic acid building block. All peptides were synthesized as free acids.

group (Table 1). Relative import efficiencies into adherently growing SKMel 37 cells were determined by flow cytometry. As reported earlier for measurements by FCM [27], import of the N-terminally labelled AntpHD analogue was about 3 times that of the corresponding MTS analogue (Fig. 1). The relative import efficiencies were highly reproducible. Except for the fluorescein-labelled A12K(Fluo) MTS analogue, fluorescent derivatization within the MTS peptide reduced the import efficiency to the levels of unspecific binding and pinocytic uptake of a fluorescein-labelled control peptide. The import efficiency of the A12K(Fluo) analogue was about 85% that of the N-terminal analogue. For the AntpHD peptide as well, one analogue (N9K(Fluo)) was imported as efficiently as the N-terminal analogue, while the other three fluorescein derivatives showed greatly reduced import efficiencies. For the Q8K(Fluo) analogue, import was reduced by 50% and nearly completely abolished for the other two analogues.

3.2. Effect of the fluorophore on import efficiency

So far, fluorescent labelling of cell-permeable peptides has been limited to the introduction of fluorophores emitting in the short-wavelength visible part of the spectrum. Fluorophores emitting in the orange to far red spectral range are highly desirable for the elimination of autofluorescence [28] and for the detection of cell-permeable peptides in the presence of GFP fusion proteins. The fluorophore tetramethylrhodamine (TAMRA) was selected based on its compatibility with solid phase peptide synthesis, price, high photostability, and spectral compatibility with fluorescein and Cy5-like dyes for simultaneous detection. A subset of TAMRA-labelled analogues was synthesized and import quantitated by flow cytometry (Fig. 2A). In contrast to fluorescein carrying one negative charge at neutral pH, TAMRA is a zwitterionic compound with a neutral net charge. Earlier analyses of structure–function relationships of cell-permeable peptides had stressed the significance of the physicochemical characteristics of the transcytosis sequence [16]. Negative, within the cell-permeable peptide charge, in particular, was shown to be incompatible with import [29]. In comparison to fluorescein, it was therefore suspected that TAMRA would significantly promote import.

As for the fluorescein analogue, full import capacity was observed for the MTS A12K(TAMRA) and AntpHD N9K(TAMRA) variants, while import was abolished for the MTS L10K(TAMRA) analogue. In contrast to the fluorescein derivatives, import of AntpHD F7K(TAMRA) was reduced by only 50%. The relative import efficiencies for the fluorescein-labelled constructs showed a strong positive correlation with those of the TAMRA-labelled constructs (Fig. 2B). In spite of the differences in the physicochemical characteristics of the dyes, derivatization of individual residues affected import in a similar way, independent of the dye. Measurements of intracellular concentrations by FCM (see below) revealed that import of the N-terminally labelled TAMRA-labelled peptides was more efficient by a factor of 1.5 than that of the respective fluorescein-labelled peptides. Considering the positive correlation of import efficiencies, the TAMRA reporter group affected import of all analogues in a similar fashion.

3.3. Concentration independence of peptide import

The concentration independence of import is a vital prerequisite for the analysis of intracellular dose–response characteristics using cell-permeable peptide cargo-constructs. To date, however, the analysis of import has been limited to the micromolar range. Recently, the authors presented intracellular concentration measurements of fluorescein-labelled cell-permeable peptides in the nanomolar

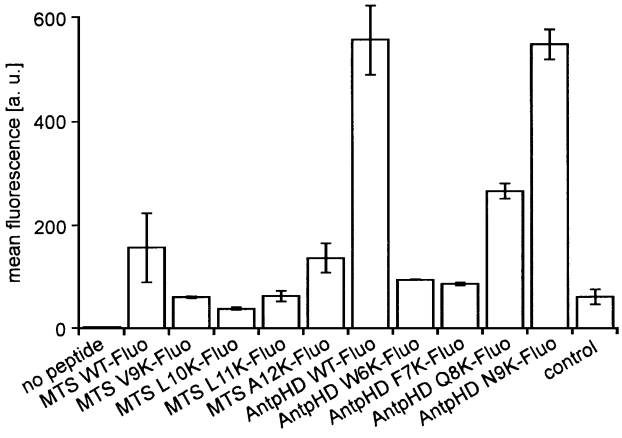

Fig. 1. Relative import efficiencies of carboxyfluorescein-labelled cell-permeable peptides. Given are the means for two independent experiments. The experiments were normalized based on the total fluorescence of both experiments. As a control for unspecific peptide binding, the amidated peptide Fluo-Ahx-SFHTMSAAKLI was used. Adherently growing SKMel 37 cells were incubated with peptides at a concentration of 4 μM at 37 °C for 2 h followed by trypsinization and flow cytometry.

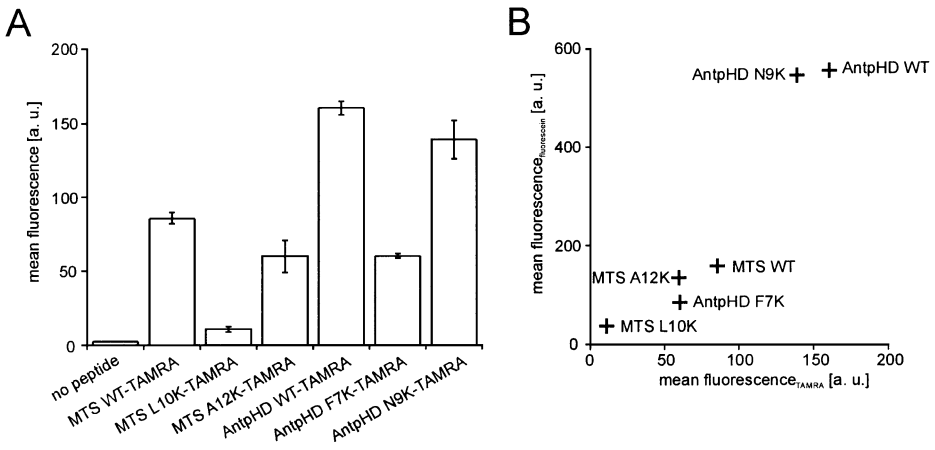

Fig. 2. Comparison of import efficiencies of carboxyfluorescein- and TAMRA-labelled cell-permeable peptide analogues. (A) Relative import efficiencies of TAMRA-labelled peptide analogues. Adherent SKMel 37 tissue culture cells were incubated with peptide at a concentration of 4 μM for 2 h, trypsinized and fluorescence measured by flow cytometry. (B) Correlation of import efficiencies of TAMRA- and carboxyfluorescein-labelled peptide analogues.

range by FCM [27]. In contrast to other techniques, FCM allows for a direct determination of molecule numbers. Due to the minuteness of the confocal detection volume (sub-femtoliter), intracellular measurements can be conducted with peptide present in the incubation buffer outside the cell.

Cellular import of analogues labelled at the N terminus was measured by FCM at lower nanomolar concentrations. Import of peptides was compared by calculating the partition coefficients as the ratio of the number of molecules in the nucleus and in the incubation buffer (Fig. 3). The TAMRA-labelled peptides were imported more efficiently than the fluorescein-labelled peptides by a factor of 1.5 (Fig. 4A). The application of FCM is limited to the subnanomolar to lower nanomolar concentration range. For this reason, it was impossible to compare the import efficiencies at nanomolar concentrations directly with those at micromolar concentrations. Instead, the relative import efficiencies of fluorescein and TAMRA-labelled MTS and AntpHD peptides determined by flow cytometry (Fig. 4B,C) were compared with those determined by FCM (Fig. 4D). The larger standard deviations of the FCM data result from the fact that this number is the ratio of the number of molecules inside and outside the cell. A total of at least 30 measurements in eight different cells were included for each condition. The higher standard deviations of the MTS peptides are explained by the stronger adsorption to the bottom of the measurement chamber which had to be compensated for in order to determine the intracellular molecule number. A detailed presentation of these intracellular concentration measurements can be found in the publication by Waizenegger et al. [27]. The relative import efficiencies determined by both methods closely matched one another. If import for either one peptide was concentration-dependent, this correspondence of import efficiencies would be very unlikely. This result, therefore, strongly supports a concentration independence of import covering the lower nanomolar to the lower micromolar range. Except for the TAMRA-labelled AntpHD analogue, all partition coefficients were significantly smaller than 1 (Fig. 4A), i.e. the concentration of the peptide inside the cells was lower than the concentration outside. The peptide concentration in the buffer therefore does not reflect the intracellular concentration; the latter depends on the peptide in each individual case.

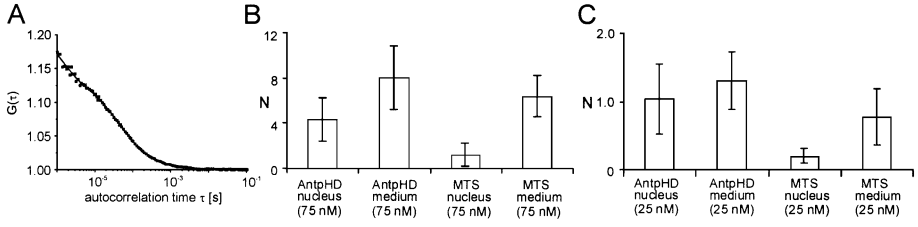

Fig. 3. Intracellular concentration measurements of cell-permeable peptides labelled at the N terminus with either fluorescein or TAMRA by fluorescence correlation microscopy. (A) Intranuclear autocorrelation measurement of Fluo-AntpHD at a concentration of peptide in the buffer of 75 nM. The amplitude of the autocorrelation function at τ=0 s is inversely related to the number of molecules in the confocal detection volume. (B, C) FCM measurements of MTS and AntpHD peptides labelled at the N terminus with either carboxyfluorescein (B) or TAMRA (C). The number of molecules were derived from two-component fits to the autocorrelation functions and corrected for fluorescence originating from molecules adsorbed to the bottom of the measurement chambers. For MTS peptides as well as for TAMRA-labelled AntpHD peptides, the molecule numbers in the buffer were lower than those of the fluorescein-labelled AntpHD peptide due to stronger adsorption of the peptides to the walls and the bottom of the measurement chamber.

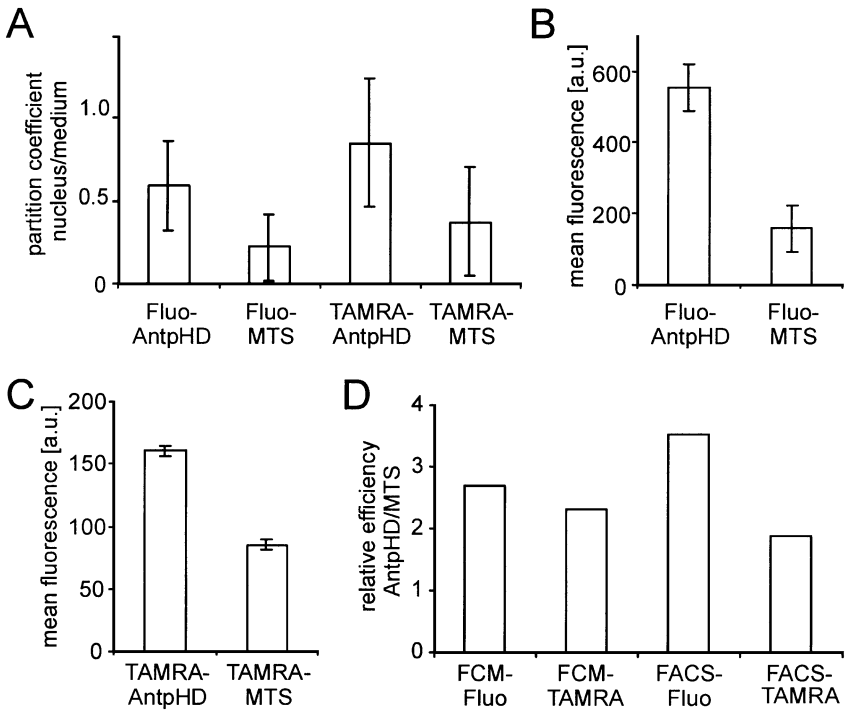

Fig. 4. Concentration independence of import. (A) FCM-derived partition coefficients of cellular uptake calculated by dividing the number of molecules in the nucleus by the number of molecules in the incubation buffer (see Fig. 3). (B, C) Import efficiencies for AntpHD and MTS peptides N-terminally labelled with either carboxyfluorescein or TAMRA. (D) Comparison of relative import efficiencies of carboxyfluorescein- and TAMRA-labelled peptides determined by FCM and flow cytometry. For FCM measurements, peptide concentrations were 75 nM for carboxyfluorescein and 25 nM for TAMRA-labelled peptides, and 2 and 4 μM for flow cytometry. In spite of these very different concentrations, the relative import efficiencies for AntpHD and MTS labelled peptides agreed with each other.

3.4. Cargo dependence of import

In order to address the cargo dependence of import for small peptide cargos in detail, N-terminally labelled AntpHD analogues with C-terminal peptide cargos of similar size were synthesized. Cargos were representative for primary applications of cell-permeable peptide constructs. These were the Myc-tag peptide EQKLISEEDL, a recently reported peptide inhibitor of the tyrosine kinase ZAP-70 KLILFLLL [30], as well as the MHC class I-

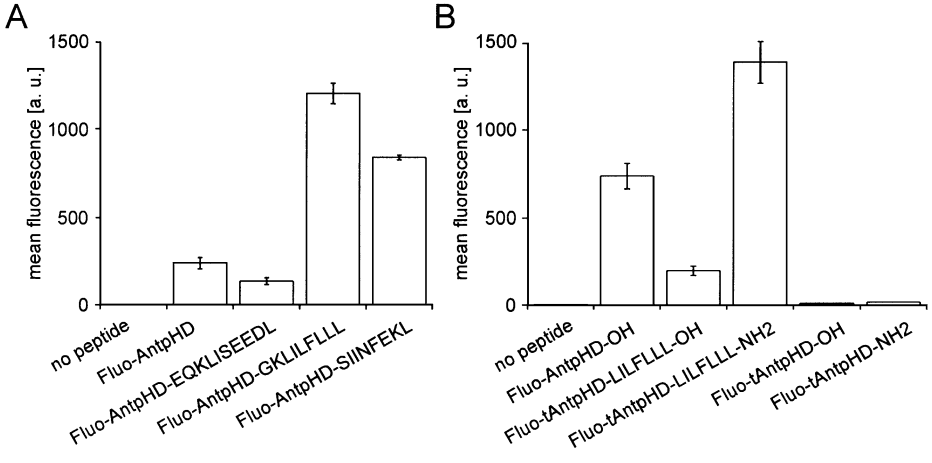

Fig. 5. Cargo dependence of import. (A) Import of AntpHD-based constructs labelled with carboxyfluorescein at the N terminus. All peptides were synthesized with a free C-terminal carboxyl-group. The N-terminal glycine residue for the ZAP-70 inhibitor peptide KLILFLLL was introduced as a spacer amino acid. (B) Dependence of import on C-terminal amidation. Constructs were based on a truncated form of the AntpHD-sequence (tAntpHD) and the import of these molecules was compared with the import efficiency of the full length AntpHD peptide. For the inhibitor construct, the C-terminal lysine residue of the import peptide served as the first residue of the inhibitor. Cells were incubated with 2 μM peptide for 2 h. Peptides were synthesized either with a free carboxyl group (-OH) or with an amidated C terminus (-NH2).

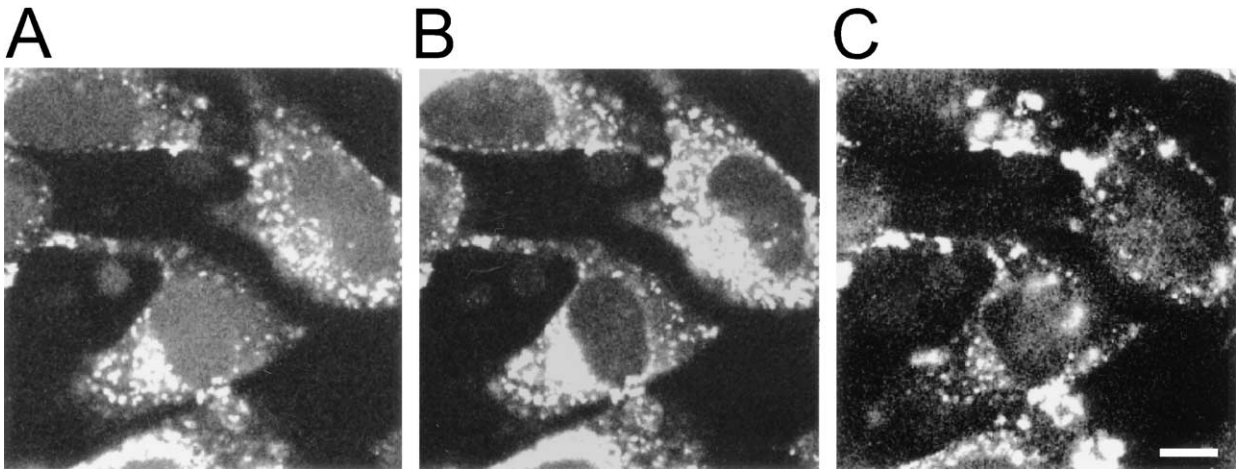

Fig. 6. Simultaneous import of three different cell-permeable peptides. Carboxyfluorescein- (A), carboxytetramethylrhodamine- (B), and S0387-labelled (C) AntpHD peptides were detected by multichannel confocal laser scanning microscopy of living cells. Cells were incubated with peptide at concentrations of 4, 1, and 2 μM for the fluorescein-, TAMRA-, and S0387-labelled peptides, respectively, for 2 h at 37 °C. The rhodamine and S0387 channel were virtually free of cross-talk as determined from control samples loaded with either fluorescein- or TAMRA-labelled peptides, alone. The bar denotes 10 μm.

binding T-lymphocyte epitope SIINFEKL [31]. The latter two have already been employed in combination with cell-permeable peptides [30,32].

Intracellular fluorescence of cells incubated with the AntpHD-ZAP-70 inhibitor peptide for 2 h was five times that of cells incubated with the AntpHD peptide, alone (Fig. 5A). In contrast to this, the C-terminal extension of the AntpHD peptide with the Myc-tag decreased the intracellular signal in comparison with the AntpHD peptide by more than 50%. For the MHC epitope, the intracellular signal was 3.5 times that of the AntpHD peptide.

The impact of minimal structural differences on import was addressed by quantitating intracellular fluorescence for pairs of peptides with either a free or amidated carboxy terminus. The constructs were based on a recently described truncated form of the AntpHD peptide comprising the C-terminal eight amino acids NRRMKWKK only [16]. Carboxy-terminal amidation had a great impact on import efficiency. Import of the amidated inhibitor construct was seven times that of the construct with the free carboxy terminus; import of the amidated truncated peptide alone was 1.5 times that of the free acid. In contrast to the initial report on the truncated peptide, in our hands this molecule only translocated weakly. The truncated penetratin peptides conjugated to the inhibitor peptide, however, exhibited a translocation efficiency similar to the efficiency of the full-length penetratin.

3.5. Triple labelling

The availability of cell-permeable peptides labelled with spectrally distinct fluorophores affords the use of combinations of different cell-permeable peptide/cargo constructs. In this way, several molecular interactions may be addressed simultaneously. To this end, only one cell-permeable peptide cargo construct carrying one specific fluorophore has been used at a time. To explore the possibility of simultaneously detecting three cell-permeable constructs in one cell, a further fluorescent analogue emitting fluorescence in the near infra-red part of the spectrum was generated. Cells were incubated with fluorescein-labelled, TAMRA-labelled, and S0387-labelled AntpHD peptides and fluorescence detected by triple-channel confocal laser scanning microscopy (Fig. 6). The cellular localization was very similar for all peptides, with a homogeneously distributed fluorescence present in the cytoplasm and in the nucleus and a vesicular pattern of fluorescence in the extra-nuclear region of the cell. In comparison to the fluorescein- and S0387-labelled peptides, the TAMRA-labelled peptide exhibited a stronger tendency to accumulate in vesicular structures.

4. Discussion

4.1. Import efficiencies of fluorescent derivatives

For both the AntpHD and the MTS peptides, an internally labelled analogue was identified with an import efficiency comparable to the efficiency of the respective N-terminally labelled peptide. All peptides were generated by solid-phase peptide synthesis. The internal label was coupled to a lysine ε-amino group with the fully side-chain protected peptide still on the solid phase. For this reason, once the peptide moiety is synthesized, the N terminus of a fully side-chain protected peptide is available for extension with a peptide cargo or non-peptide cargo molecule. Based on this labelling strategy, we anticipate that fluorescently labelled cell-permeable peptides will gain significance as pharmacokinetic modifiers for small molecules that otherwise do not penetrate the plasma membrane. The fluorophore serves as the reporter group to quantitate import of the small molecule.

Import of AntpHD analogues was more efficient than import of MTS analogues by a factor of about 3. So far, only few reports exist in which the import competence of different sequence motifs were compared relative to each other. To our knowledge, no direct comparison of a purely aliphatic motif, such as the MTS peptide, and an amphipatic motif, such as the AntpHD peptide, has been presented so far. One should note, however, that in our case a polyethyleneglycol building block was introduced at the C terminus of the MTS peptide in order to increase the solubility in aqueous buffers. In our hands, AntpHD is the preferred cell-permeable tag because of the higher import efficiency, higher solubility in aqueous buffers, and comparatively less adsorption to the walls of the measurement chambers [27].

By introduction of fluorophores within the central part of the peptide sequence, constructs carrying a second fluorophore either within the cargo or at the amino terminus of the cargo may be generated. Such constructs may serve as cell-permeable probes for intracellular protease activity by detection of FRET [20] with optimised FRET characteristics.

The abrogation of import of the AntpHD W6K(Fluo) construct further substantiates the role of this specific side-chain for import [2]. Interestingly, replacement with the large aromatic fluorophore moiety had the same effect as replacement with alanine. While import competence may be conveyed by the general physicochemical characteristics of the whole peptide [17], efficient uptake is strongly dependent on the presence of structural determinants at specific positions of the cell-permeable peptide. The finding that only one out of four analogues derivatized with bulky fluorophores at consecutive positions entered cells efficiently supports the view that these peptides associate to a biological membrane in a well-defined orientation [18,33].

The import efficiencies for AntpHD as well as for MTS peptides labelled with the negatively charged carboxyfluorescein strongly correlated with those labelled with the zwitterionic carboxytetramethylrhodamine. This finding supports the notion that for these two cell-permeable peptide motifs, efficient uptake is a function of the peptide structure rather than the overall physicochemical characteristic. Surprisingly, the introduction of the negatively charged fluorescein moiety in the central part of the strongly basic AntpHD peptide was compatible with efficient import. Recently, we introduced FCM for a direct determination of intracellular peptide concentrations [27]. The determination of partition coefficients for cellular uptake by FCM revealed that import of the TAMRA-labelled analogues was slightly more efficient than that of the fluorescein-labelled analogues. From the correlation of import efficiencies, we conclude, however, that this effect must be very similar for all TAMRA derivatives. Comparison of the cellular distribution of fluorescein and TAMRA-labelled peptides having no intrinsic transcytosis capacity has shown that the TAMRA label may promote accumulation of these peptides in vesicular structures by itself (own unpublished data). This dye-dependent contribution may provide an explanation for the generally higher import efficiency of TAMRA- over fluorescein-labelled peptides. One should note that FCM is the only technique capable of comparing import efficiencies for cell-permeable substances labelled with different fluorophores that does not depend on cumbersome calibration procedures to normalize on the detection efficiencies for different spectral ranges.

Comparison of import efficiencies determined by FCM and flow cytometry demonstrated a concentration independence of import from the lower micromolar to the lower nanomolar range. So far, the analysis of the concentration dependence of import has been limited from the higher nanomolar to the mean micromolar range [1,16,34]. That large of a concentration range could only be covered by combination of these two analytical techniques. Flow cytometry is too insensitive to quantitate import in the lower nanomolar range, while FCM can only be used at lower nanomolar concentrations.

To further extend the spectral range of fluorescent cell-permeable peptide derivatives, an N-terminal analogue of AntpHD labelled with the indocyanine dye S0387 was generated and the intracellular distribution investigated with confocal laser scanning microscopy. In this way we were able to demonstrate the simultaneous detection of three different cell-permeable peptide constructs. In all cases, a vesicular staining was present that was the most pronounced for the TAMRA-labelled derivative. So far the nature of the vesicles has not been addressed. The vesicular accumulation of the cell-permeable peptides will present some limitation in the analysis of a cargo-driven subcellular targeting of cell-permeable peptide constructs, e.g. to target molecules present in specific cellular compartments. Our data indicate that TAMRA will be the least suited fluorophore for this kind of application.

4.2. Cargo dependence of import

The independence of import on the nature of the fluorophore was contrasted by a marked dependence on the peptide cargo. Even though all peptides investigated were very similar in size, ranging from 8 to 10 amino acids, import efficiencies varied by as much as a factor of 8 for the Myc-tag peptide and for the ZAP-70 inhibitor peptide. While the Myc-tag peptide is characterised by a negative net charge of three, the AntpHD-conjugated SIINFEKL-epitope carries one negative net charge, and the ZAP-70 inhibitor peptide is strongly hydrophobic with a neutral net charge. Apparently, a strong negative correlation exists between the negative charge of the cargo and the import efficiency. So far, this observation has only been reported for the transcytosis sequence alone [29]. The effect that subtle structural changes may have on uptake was addressed using a truncated analogue of the penetratin peptide. For the import peptide alone, as much as for a conjugate with the ZAP-70 inhibitor peptide, C-terminal amidation greatly

affected the import efficiency. Interestingly, the truncated peptide alone was very inefficient in cellular import when compared with the full-length penetratin peptide. Previously, this peptide was shown to possess about 60% of the translocation efficiency of the full-length Antennapedia peptide [16]. This discrepancy may result from the differences in assay conditions. In the initial report, a biotinylated analogue was employed and cells were labelled with fluorescently labelled streptavidin after fixation. Fixation, however, may lead to a loss of small molecules due to poor cross-linking. It cannot be excluded that only a fraction of molecules, e.g. those present in vesicular structures, were retained in the cells while the major part of those present in the cytoplasm and nucleus were lost. For this reason, all analyses in our report were based on fluorescence in living cells. In one of the initial analyses of the structure–activity relationship of the AntpHD peptide, Derossi et al. [2] reported that removal of only two amino acids from either the N or C terminus abolished import. For the truncated peptide, import competence was rescued by conjugation of the highly hydrophobic cargo KLILFLLL. It seems that in this case, the cargo complements the cell-permeable moiety.

In summary, attachment of fluorophores that differed markedly in their physicochemical characteristics was tolerated surprisingly well. Import depended much more on the cargo than on the nature of the fluorophore. Analysis of import by FCM revealed that the concentrations in the buffer did not reflect the concentrations of these molecules inside the cells. Both findings stress the need for a detailed monitoring of intracellular peptide concentrations in applications such as the analysis of structure function relationships and quantitatively controlled inhibition of intracellular processes. The fluorescent peptide analogues identified in this study provide the tools for that purpose.

Acknowledgements

We thank Günther Jung for generous support in solid phase chemistry and instrumental analytics and Nicole Sessler for expert technical assistance in peptide synthesis. The SKMel 37 cells were obtained from Stefan Stevanović. R.B. was a recipient of a postdoctoral fellowship from the Volkswagen Foundation.

References

[1] D. Derossi, S. Calvet, A. Trembleau, A. Brunissen, G. Chassaing, A. Prochiantz, J. Biol. Chem. 271 (1996) 18188–18193.
[2] D. Derossi, A.H. Joliot, G. Chassaing, A. Prochiantz, J. Biol. Chem. 269 (1994) 10444–10450.
[3] Y.-Z. Lin, S. Yao, R.A. Veach, T.R. Torgerson, J. Hawiger, J. Biol. Chem. 270 (1995) 14255–14258.
[4] S. Dokka, D. Toledo-Velasquez, X. Shi, L. Wang, Y. Rojanasakul, Pharm. Res. 14 (1997) 1759–1764.
[5] M.-P. Schutze-Redelmeier, H. Gournier, F. Garcia-Pons, M. Moussa, A.H. Joliot, M. Volovitch, A. Prochiantz, F.A. Lemonnier, J. Immunol. 157 (1996) 650–655.
[6] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, Science 285 (2000) 1569–1572.
[7] J. Hawiger, Curr. Opin. Chem. Biol. 3 (1999) 89–94.
[8] A. Prochiantz, Curr. Opin. Neurobiol. 6 (1996) 629–634.
[9] M. Rojas, J.P. Donahue, Z. Tan, Y.-Z. Lin, Nat. Biotechnol. 16 (1998) 370–375.
[10] R.W. Wallace, Drug Discov. Today 2 (1997) 124–125.
[11] M. Pooga, U. Soomets, M. Hallbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.X. Hao, X.J. Xu, Z. Wiesenfeldhallin, T. Hokfelt, A. Bartfai, U. Langel, Nat. Biotechnol. 16 (1998) 857–861.
[12] M. Rojas, S. Yao, Y.-Z. Lin, J. Biol. Chem. 271 (1996) 27456–27461.
[13] X.-Y. Liu, S. Timmons, Y.-Z. Lin, J. Hawiger, Proc. Acad. Natl. Sci. U. S. A. 93 (1996) 11819–11824.
[14] L. Zhang, T.R. Torgerson, X.-Y. Liu, S. Timmons, A.D. Colosia, J. Hawiger, J.P. Tam, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9184–9189.
[15] A. Schulz, K. Adermann, M. Eulitz, S.M. Feller, C. Kardinal, Tetrahedron Lett. 56 (2000) 3889–3891.
[16] P.M. Fischer, N.Z. Zhelev, S. Wang, J.E. Melville, R. Fåhraeus, D.P. Lane, J. Pept. Res. 55 (2000) 163–172.
[17] A. Scheller, B. Wiesner, M. Melzig, M. Bienert, J. Oehlke, Eur. J. Biochem. 267 (2000) 6043–6049.
[18] G. Drin, M. Mazel, P. Clair, D. Mathieu, M. Kaczorek, J. Temsamani, Eur. J. Biochem. 268 (2001) 1304–1314.
[19] S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda, Y. Sugiura, J. Biol. Chem. 276 (2001) 5836–5840.
[20] R.M. Clegg, Curr. Opin. Biotechnol. 6 (1995) 103–110.
[21] Y. Nagai, M. Miyazaki, R. Aoki, T. Zama, S. Inouye, K. Hirose, M. Iino, M. Hagiwara, Nat. Biotechnol. 18 (2000) 313–314.
[22] J. Jones, R. Heim, E. Hare, J. Stack, B.A. Pollok, J. Biomol. Screen. 5 (2000) 307–317.
[23] R. Brock, G. Vàmosi, G. Vereb, T.M. Jovin, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 10123–10128.
[24] P.L. Southwick, L.A. Ernst, E.W. Tauriello, S.R. Parker, R.B. Mujumdar, S.R. Mujumdar, H.A. Clever, A.S. Waggoner, Cytometry 11 (1990) 418–430.
[25] F. Perez, P.-M. Lledo, D. Karagogeos, J.-D. Vincent, A. Prochiantz, J. Ayala, Mol. Endocrinol. 8 (1994) 1278–1287.
[26] B. Fleckenstein, G. Jung, K.-H. Wiesmüller, Semin. Immunol. 11 (1999) 405–416.
[27] T. Waizenegger, R. Fischer, R. Brock, Biol. Chem. 383 (2002) 291–299.
[28] J.E. Aubin, J. Histochem. Cytochem. 27 (1979) 36–43.
[29] A. Scheller, J. Oehlke, B. Wiesner, M. Dathe, E. Krause, M. Beyermann, M. Melzig, M. Bienert, J. Pept. Sci. 5 (1999) 185–194.
[30] K. Nishikawa, S. Sawasdikosol, D.A. Fruman, J. Lai, Z. Songyang, S.J. Burakoff, M.B. Yaffe, L.C. Cantley, Mol. Cell 6 (2000) 969–974.
[31] O. Rötzschke, K. Falk, S. Stevanović, G. Jung, P. Walden, H.-G. Rammensee, Eur. J. Immunol. 21 (1991) 2891–2894.
[32] G.A. Pietersz, W.J. Li, V. Apostolopoulos, Vaccine 19 (2001) 1397–1405.
[33] C. Du, S. Yao, M. Rojas, J. Pept. Res. 51 (1998) 235–243.
[34] D.J. Mitchell, D.T. Kim, L. Steinman, C.G. Fathman, J.B. Rothbard, J. Pept. Res. 56 (2000) 318–325.