

# Cationic cell-penetrating peptides interfere with TNF signalling by induction of TNF receptor internalization

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## Summary

Cationic cell-penetrating peptides (CPPs) have been used widely as delivery vectors for the import of molecules that otherwise do not cross the plasma membrane of eukaryotic cells. In this work, we demonstrate that the three cationic CPPs, Antennapedia homeodomain-derived peptide (Antp), nona-arginine and Tat-derived peptide, inhibit tumour necrosis factor (TNF)-mediated signal transduction. This inhibition is based on the downregulation of TNF receptors at the cell surface by induction of internalization. In contrast to TNF-dependent receptor internalization, no receptor activation occurs. The receptor downregulation is not restricted to the CPPs. Remarkably, the HIV-1 Tat protein itself also induces the internalization of TNF receptors. The dynamin dependence of the internalization, as well as the fact that epidermal growth factor receptors are also internalized, suggest a general induction of clathrin-dependent endocytosis as the mechanism of action. The significance of these findings for

the use of cationic CPPs in the import of bioactive peptides is demonstrated here using a conjugate consisting of Antp and a Smac protein-derived cargo peptide. The cargo alone, when introduced into cells by electroporation, enhanced TNF-induced apoptosis by inhibiting the anti-apoptotic action of IAPs (inhibitor of apoptosis proteins). For the Antp-Smac conjugate at concentrations below 40  $\mu$ M the inhibitory effect of the Antp peptide compensated for the pro-apoptotic activity of the cargo, and led to the protection of cells against TNF-mediated apoptosis. These data provide important new information for the use of cationic CPPs for the cellular delivery of bioactive molecules.

Key words: Antennapedia homeodomain, cell-penetrating peptides, epidermal growth factor receptor, HIV-1 Tat protein, receptor internalization, tumour necrosis factor

## Introduction

The import of membrane-impermeable peptides and nucleotides into mammalian cells is becoming increasingly used for the selective interference with cellular signal transduction. The import strategies presented so far can be subdivided into those that transiently permeabilize the plasma membrane, such as electroporation and streptolysine O treatment, and those employing carrier molecules that enter the cell while maintaining membrane integrity (Stephens and Pepperkok, 2001). Among the latter, cell-penetrating peptides (CPPs) have become established as a powerful tool for a non-invasive cellular delivery of peptides, oligonucleotides and even nanoparticles (Langel, 2002; Fischer et al., 2001).

Three highly basic import peptides, the *Drosophila* Antennapedia homeodomain-derived Antp peptide (Derossi et al., 1994), the human immunodeficiency virus-1 (HIV-1) Tat-derived Tat peptide (Vives et al., 1997) and oligo-arginine peptides (Mitchell et al., 2000) have been applied successfully to deliver cargoes into mammalian cells (May et al., 2000; Ye et al., 2002). Recently, endocytosis has been identified as a major route for uptake of cationic CPPs (Richard et al., 2003; Drin et al., 2003). It has been shown (i) that translocation of

CPPs from vesicular compartments into the cytoplasm requires acidification of endosomes (Fischer et al., 2004; Potocky et al., 2003) and (ii) that retrograde transport may also be involved in the trafficking and release into the cytoplasm (Fischer et al., 2004).

Evidence is accumulating that release of CPP-cargo conjugates into the cytoplasm is a bottleneck for the interaction of cargo peptides with cytoplasmic target proteins (Caron et al., 2004; Sengoku et al., 2004). For this reason we compared CPP-mediated import with electroporation as an alternative import strategy. Electroporation induces a transient and reversible permeabilization of the plasma membrane thereby bypassing the endocytic pathway (Gehl, 2003). We performed these experiments in the context of tumour necrosis factor (TNF)-dependent signal transduction. TNF mediates cellular responses via two distinct TNF receptors TNF-R1 and TNF-R2. In HeLa cells TNF-R1-dependent signalling either leads to the activation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) and c-jun or, in the presence of cycloheximide, to the induction of apoptosis (Wajant et al., 2003). The NF- $\kappa$ B activation cascade has been successfully interrupted by short peptides, introduced into cells using CPPs (May et al., 2000; Swaroop et al., 2001). Similarly,

it was reported that apoptosis can be enhanced by delivery of pro-apoptotic cargo peptides coupled to CPPs (Fulda et al., 2002; Holinger et al., 1999).

As a bioactive peptide we selected a peptide corresponding to the seven N-terminal amino acids of the Smac (second mitochondria-derived activator of caspase) protein (Wu et al., 2000). Smac is a pro-apoptotic protein, that, following an apoptotic stimulus, is released from mitochondria and inhibits the anti-apoptotic action of IAPs (inhibitor of apoptosis proteins) (Du et al., 2000). Multiple IAP molecules including c-IAP1, c-IAP2 and XIAP have been shown to bind and block the activity of the apoptotic proteases caspase-3 and -7 (Deveraux et al., 1997). Smac binds to the hydrophobic pocket of IAP with its four amino-terminal residues (Ala-Val-Pro-Ile) and thereby prevents the binding of IAPs to caspases (Wu et al., 2000). Recently, it has been also shown that Smac selectively causes the rapid degradation of c-IAP1 and c-IAP2 and enhancement of apoptosis in HeLa cells (Yang and Du, 2004). A synthetic Smac-derived peptide exerts the same effects (Fulda et al., 2002).

Our analyses revealed that import of the pro-apoptotic peptide by electroporation yielded a much higher activity than CPP-mediated import. To our surprise, this increase in activity was not due to the sequestration of CPP-cargo constructs in the endosomal compartment, alone. We demonstrate that all three widely used cationic CPPs Antp, Tat and nona-arginine (R9) inhibited TNF receptor-dependent signalling. We explain the molecular basis for this observation by demonstrating that the CPPs induce the internalization of TNF receptors in a dynamin-dependent but receptor activation-independent fashion.

## Materials and Methods

### Cells and reagents

The human cervical carcinoma cell line HeLa was obtained from the American Type Culture Collection (Manassas, VA, USA). The human myelomonocytic cell line THP-1 (Tsuchiya et al., 1980) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). HeLa cells stably transfected with TNF-R2 (HeLa-TNF-R2) and recombinant TNF mutants with specificity for TNF-R1 (Cys-TNF32W/86T) and with specificity for TNF-R2 (Cys-TNF143N/145R) have been described elsewhere (Weiss et al., 1997). HeLadynK44A expressing a dominant negative form of dynamin-2 under the control of a tet on/off promoter, cultured in medium containing 1 µg/ml tetracycline were a kind gift from Bo van Deurs (University of Copenhagen, Denmark). The expression of mutant dynamin was induced by tetracycline deprivation for at least 24 hours. All cell lines were maintained in RPMI 1640 medium with stabilized glutamine and 2.0 g/l NaHCO<sub>3</sub> (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (PAN Biotech). Fluorogenic caspase-3 substrate (Ac-DEVD-AMC) was obtained from Calbiochem (Bad Soden, Germany). The monoclonal murine antibodies anti-human TNF-R1 (H398) and anti-human TNF-R2 (80M2) have been described elsewhere (Thoma et al., 1990). The mouse monoclonal antibody against the p65 subunit of human NF-κB was from Santa Cruz Biotechnology (Heidelberg, Germany), the mouse monoclonal antibody anti-human Fas (clone 555671) was from Pharmingen (Hamburg, Germany) and the mouse monoclonal antibody (425) anti-human EGFR was from Merck (Darmstadt, Germany). The secondary goat anti-mouse Cy5-labelled antibody was obtained from Dianova (Hamburg, Germany). The Zenon Mouse IgG1

Labeling Kit (specific for the Fc part of IgG1 antibodies) and the transferrin Alexa Fluor 633 conjugate were purchased from Mobitech (Göttingen, Germany). Bafilomycin A1 was from Tocris Biotrend (Bristol, UK), TAPI-1 was from Calbiochem, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and methyl-β-cyclodextrin (MβCD) were obtained from Sigma (Deisenhofen, Germany).

### Peptide synthesis

Automated peptide synthesis was performed by solid-phase Fmoc/tBu chemistry using an automated peptide synthesizer for multiple peptide synthesis (RSP5032, Tecan, Hombrechtikon, Switzerland) in 2 ml syringes according to a protocol described elsewhere (Fischer et al., 2004). Smac, Smac-Antp, Rev and Rev-Antp (Table 1) were synthesized using a N<sup>α</sup>-carboxyfluorescein-labelled lysyl-Rink amide resin (Fischer et al., 2003). The purity of all peptides was determined by analytical high-performance liquid chromatography (HPLC). The identity of the peptides was confirmed by matrix-assisted laser desorption/ionization – time of flight mass spectroscopy (MALDI-TOF MS) analysis. Peptides with a purity of less than 85% were purified by preparative HPLC. Purity of all peptides used was >95% (214 nm HPLC).

### Labelling of recombinant HIV-1 Tat protein

The recombinant Tat protein, in which all cysteine residues were replaced with alanines, was obtained from P. Rösch (University of Bayreuth, Germany). The lyophilized protein (2 mg) was dissolved in 1 ml buffer (10 mM K<sub>3</sub>PO<sub>4</sub>, 150 mM NaCl, pH 6.4) and reacted with fluorescein isothiocyanate (0.15 mg; Fluka, Deisenhofen, Germany) with a 3 M excess of the fluorophore for 5 hours. The unreacted free fluorophore was separated from the labelled Tat protein on a gel permeation column (PD10, Amersham Biosciences, Freiburg, Germany) using a 100 mM triethyl ammonium acetate buffer pH 7.5 for elution of the protein. The FITC-labelled Tat protein was then concentrated to 80 µM using a Centricon with a cut-off of 3 kDa (YM 3, Millipore, Schwallbach, Germany), essentially as described by the supplier.

### Flow cytometry

HeLa cells were seeded at a density of 5 × 10<sup>4</sup>/well in 24-well plates (Sarstedt, Nümbrecht, Germany) in serum-containing RPMI 1640. One day later, the cells were washed with medium and incubated in RPMI 1640, containing peptides in the appropriate concentrations for 30 minutes. After incubation, cells were washed with medium, detached by trypsinization for 5 minutes, resuspended in PBS and measured immediately by flow cytometry (BD FACSCalibur System, Becton Dickinson, Heidelberg, Germany). For the determination of peptide loading in electroporated cells, cells were washed twice with medium after electroporation, then resuspended in PBS and analysed immediately by flow cytometry. In each case, the fluorescence of 1 × 10<sup>4</sup> vital cells was measured. Vital cells were gated based on sideward and forward scatter.

### Analysis of intracellular peptide distribution by confocal laser scanning microscopy

Confocal laser scanning microscopy was performed on an inverted LSM 510 laser scanning microscope (Carl Zeiss, Göttingen, Germany) using a Plan-Apochromat 63 × 1.4 lens. All measurements of peptide uptake were performed with living, non-fixed cells. HeLa cells were seeded at a density of 4 × 10<sup>4</sup>/well in eight-well chambered cover glasses (Nunc, Wiesbaden, Germany). One day later, cells were washed once with medium, followed by incubation with medium containing the respective peptide. After incubation, cells were

washed with medium and images were acquired immediately. For detection of fluorescein-labelled peptides the 488 nm line of an argon ion laser was directed over an HFT UV/488 beam splitter and fluorescence was detected with a BP 505-550 band pass filter. For double detection of fluorescein-labelled peptides and Alexa Fluor 633-labelled transferrin or Alexa Fluor 647-labelled antibodies the 488 nm line of the argon ion laser and the light of a 633 nm helium/neon laser were directed over an HFT UV/488/633 beam splitter and fluorescence was detected using an NFT 545 beam splitter in combination with a BP 505-530 band pass filter for fluorescein detection and an LP 650 long pass filter for Alexa Fluor 633 and Alexa Fluor 647 detection.

### Electroporation

HeLa cells were harvested with trypsin/EDTA (ethylenediaminetetraacetic acid: Biochrom, Berlin, Germany), resuspended in medium containing the indicated peptides and then electroporated in 4 mm cuvettes at a density of  $2 \times 10^6$  cells/ml, using a 15-msecond pulse of 330 V and 1700  $\mu$ F maximal resistance (Fischer Electroporator, Heidelberg, Germany). After electroporation, cells were incubated in the cuvettes for a further 30 minutes, washed twice with medium and seeded at a density of  $5 \times 10^5$  cells per well in 6-well plates, for further analysis.

### Caspase-3 activity assay

HeLa cells were incubated with medium containing peptides for 30 minutes at 37°C. After washing, cells were treated as indicated with TNF and cycloheximide (CHX) for the induction of apoptosis followed by incubation for a further 6 hours. Cells were harvested by scraping, washed with ice-cold PBS and lysed in lysis buffer [1% Triton X-100, 150 mM NaCl, pH 7.7, supplemented with protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany)] for 30 minutes on ice. The protein content in lysates was determined using a commercially available Bradford protein assay kit (BioRad Laboratories, München, Germany). Equivalents of 30  $\mu$ g protein for each sample were diluted in caspase activity buffer (20 mM Hepes, 10 mM dithiothreitol, 10% glycerol, 100 mM NaCl, pH 7.5) to a total volume of 120  $\mu$ l. Caspase-3 substrate was added to the samples to a final concentration of 2  $\mu$ M. The efficiency of the substrate cleavage by active caspase-3 was analysed using a luminescence spectrometer LS50B (PerkinElmer, Norwalk, CT, USA) immediately after substrate addition and after 1 hour incubation at 37°C.

### Cytotoxicity assay

HeLa cells were seeded in 96-well microtiter plates ( $1.5 \times 10^4$  cells/well) and cultivated overnight. The next day cells were treated with peptides for 30 minutes at 37°C, washed and stimulated with the TNF-R1-specific TNF mutant (100 ng/ml) and CHX (2  $\mu$ g/ml) for the induction of apoptosis. In the other group, cells were electroporated with increasing amounts of peptides, then seeded in a 96-well microtiter plate and immediately stimulated with TNF/CHX. After an additional 24-hour incubation at 37°C, cells in both groups were washed with PBS followed by staining with crystal violet [20% (v/v) methanol, 0.5% (w/v) crystal violet] for 15 minutes. The wells were washed with H<sub>2</sub>O and air-dried. The dye was dissolved in methanol for 15 minutes and the optical density at 550 nm was measured with an ELISA plate reader (Molecular Devices SpectraMax 340, GMI, USA).

### Enzyme-linked immunosorbent assay (ELISA)

HeLa cells were treated as indicated and the corresponding cell-culture supernatants were analysed by a human IL-8-specific ELISA (Pharmingen) according to the manufacturer's protocol.

### MTT assay

After removing 120  $\mu$ l of the supernatant for ELISA analysis, cells were incubated with MTT at a concentration of 1 mg/ml for 4 hours. The formazan product was solubilized with SDS [10% (w/v) in 10 mM HCl]. Cell viability was determined by measuring the absorbance of each sample at 570 nm using the microplate reader.

### Immunofluorescence

HeLa cells were seeded at a density of  $4 \times 10^4$ /well in 8-well chambered cover glasses (Nunc, Wiesbaden, Germany) and incubated for 16 hours. After TNF stimulation as described above, cells were washed with medium and then fixed with 3% paraformaldehyde in HBS (Hepes-buffered saline, 10 mM Hepes, 150 mM NaCl, pH 7.2) for 10 minutes at room temperature (RT), followed by washing and permeabilization with HBS containing 0.1% saponin (Sigma) and 0.1% (w/v) BSA (saponin buffer). Intracellular staining with monoclonal anti-human p65 IgG (3  $\mu$ g/ml) was carried out in the saponin buffer for 1 hour at room temperature. The cells were washed twice with saponin buffer followed by incubation with a Cy5-labelled goat anti-mouse secondary antibody at 1:500 dilution in saponin buffer for 30 minutes at RT. After washing twice for 5 minutes with saponin buffer, medium was added to the cells and samples were analysed by confocal microscopy.

### Receptor internalization assay

Cells were incubated with medium containing peptides or control medium without peptides for 30 minutes, washed with medium, harvested with trypsin/EDTA (0.05/0.02% w/v) and washed with ice-cold PBS. Then cells were incubated with the indicated primary antibody (5  $\mu$ g/ml) for 1 hour on ice, washed with ice-cold PBS and incubated for further 30 minutes on ice with the secondary goat anti-mouse Cy5-labelled antibody, diluted 1:500.

## Results

### Conjugation of the pro-apoptotic Smac-derived peptide to Antp reduces the bioactivity of the Smac peptide

To investigate the dependence of the activity of peptides interfering with cellular signal transduction on the mechanism of cellular import the following peptides were synthesized: (i) Smac-Antp, consisting of the seven N-terminal amino acids (AVPIAQK) from the Smac protein at the N terminus and the Antp-derived peptide at the C terminus and (ii) a peptide comprising the Smac sequence only (Table 1; entries 1 and 2). The first peptide served for the analysis of CPP-mediated import, the second peptide was introduced into cells via electroporation. Peptides with the reverse Smac sequence were synthesized as controls (Table 1; entries 3 and 4). All peptides contained a fluorescein-labelled lysine residue at the C terminus in order to determine the cellular uptake and the subcellular distribution by flow cytometry and fluorescence microscopy. In a first step, the concentration dependence of

**Table 1. CPPs used in this study**

Entry	Peptide	Sequence
1	Smac-Antp	AVPIAQK-RQIKIWFQNRRMKWKK- $\epsilon$ K(Fluo)-CONH <sub>2</sub>
2	Smac	AVPIAQK- $\epsilon$ K(Fluo)-CONH <sub>2</sub>
3	Rev-Antp	KQAIPVA-RQIKIWFQNRRMKWKK- $\epsilon$ K(Fluo)-CONH <sub>2</sub>
4	Rev	KQAIPVA- $\epsilon$ K(Fluo)-CONH <sub>2</sub>
5	Antp	Fluo-RQIKIWFQNRRMKWKK-CONH <sub>2</sub>
6	R9	Fluo-RRRRRRRRR-CONH <sub>2</sub>
7	Tat	Fluo-YGRKKRRQRRR-CONH <sub>2</sub>

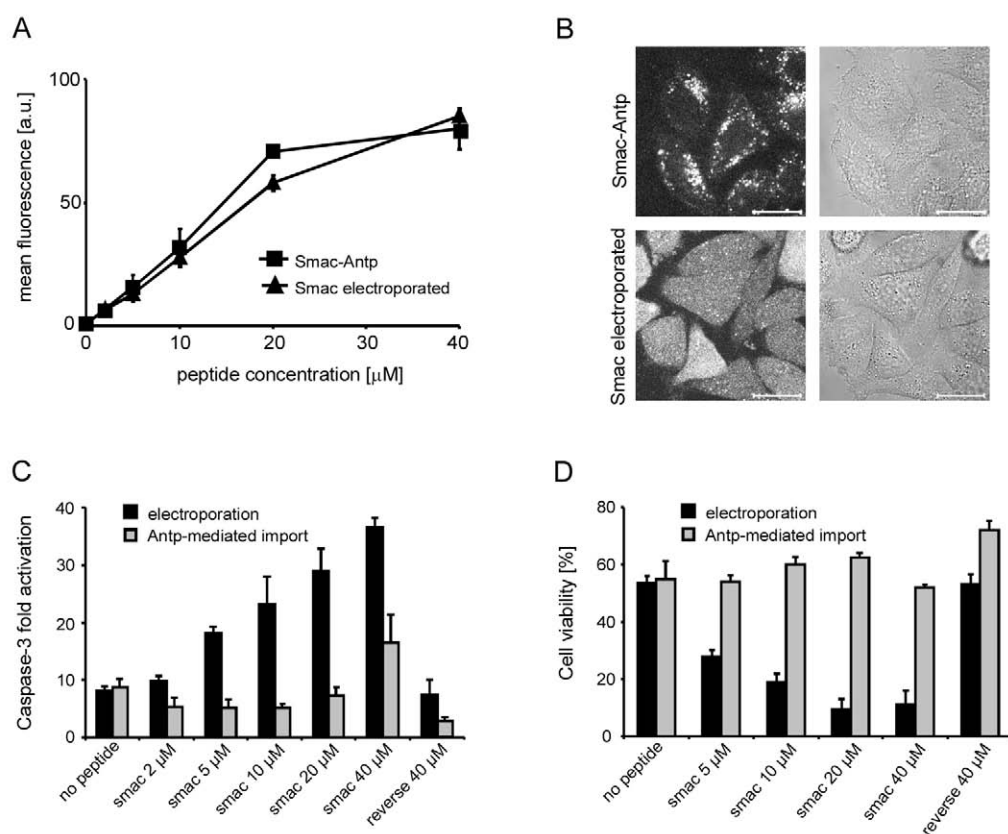


both import strategies was compared using flow cytometry. Optimized parameters were selected for the electroporation of HeLa cells. Both strategies were able to deliver peptides into cells with similar efficiency (Fig. 1A). However, live cell fluorescence microscopy revealed a rather different subcellular localization of peptides introduced by the two approaches (Fig. 1B). Antp-mediated import resulted in a vesicular enrichment of peptides with only weak fluorescence in the cytoplasm, consistent with the import of peptides by endocytosis (Fischer et al., 2004; Drin et al., 2003). In contrast, import of peptides via electroporation resulted in a homogenous cytoplasmic and nuclear distribution of the peptide.

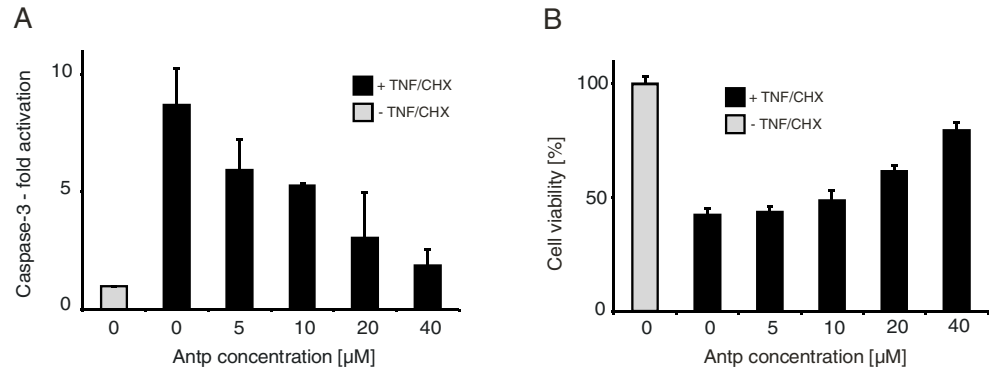
To test the implications of the different subcellular localization of peptides on their bioactivity, HeLa cells were either incubated or electroporated with increasing concentrations of Smac-Antp or Smac prior to the induction of apoptosis. Apoptosis was induced by incubation of cells with TNF in combination with CHX. The induction of apoptosis strongly correlates with the activation of effector caspases (Fernandes-Alnemri et al., 1994). The Smac protein modulates the apoptotic signalling cascade by eliminating the inhibitory effect of IAPs on caspase activity. For this reason, we first performed a caspase-3 activity assay to determine the effect of the Smac-Antp and Smac peptides on the TNF/CHX-induced caspase activity (Fig. 1C). The Smac peptide

introduced into cells via electroporation potentiated the TNF/CHX-dependent induction of caspase-3 activity in a concentration-dependent manner. In contrast, for the Antp-conjugated peptide an enhancement of caspase-3 activity was observed only at a concentration of 40  $\mu$ M. Interestingly, the incubation of cells with the Smac-Antp conjugate at lower concentrations (2–20  $\mu$ M) led to the opposite response: the inhibition of caspase-3 activity. To confirm that the enhancement of caspase-3 activity at 40  $\mu$ M Smac-Antp was not due to a cytotoxic effect, cells were pre-incubated with the control peptide Rev-Antp containing the reverse, inactive KQAIPVA sequence. In this case, the inhibition of caspase-3 activity even exceeded the one observed for Smac-Antp at lower concentrations. To validate the effects of the peptides on the TNF/CHX-mediated induction of apoptosis, we performed cell viability assays (Fig. 1D). The results of these experiments were fully consistent with the observations obtained for the caspase-3 activity assay. In summary, the results indicate that the two functional domains of the Smac-Antp conjugate exert two competing activities on the TNF/CHX-dependent induction of caspase-3 activity and apoptosis. While the Smac moiety leads to the expected enhancement of the TNF/CHX-mediated apoptotic response, the Antp moiety seems to be associated with an, as yet, undocumented inhibitory effect.

**Fig. 1.** Effect of the uptake mechanism on the cargo bioactivity. (A) HeLa cells were either incubated with Smac-Antp for 30 minutes at 37°C or electroporated with Smac with increasing concentrations of each peptide, harvested with trypsin/EDTA and analysed by flow cytometry. Error bars represent the standard deviation of the mean of three independent experiments (a.u., absorbance units). (B) HeLa cells were either incubated or electroporated with medium containing Smac-Antp or Smac (each 5  $\mu$ M) and analysed by laser scanning microscopy. The left panels show fluorescein fluorescence, the right panels are transmission images. Scale bars: 20  $\mu$ m. (C) HeLa cells were either incubated with increasing concentrations of Smac-Antp or with Rev-Antp for 30 minutes at 37°C or electroporated with Smac or Rev peptides. After removal of peptides cells were stimulated with the TNF-R1-specific mutant TNF (100 ng/ml) and CHX (2  $\mu$ g/ml) for 6 hours at 37°C, then scraped off the surface, washed and lysed. Caspase-3 activity was determined in cell lysates using a fluorogenic caspase-3 substrate and expressed as fold activation by dividing the caspase-3 activity in treated cells by the activity in untreated cells (no peptide, no TNF/CHX). Error bars represent the standard deviation of the mean of three independent experiments. (D) Cells were either incubated or electroporated with medium containing peptides. After washing, cells were treated, in triplicate, for 24 hours at 37°C with TNF-R1-specific mutant TNF (100 ng/ml) in the presence of CHX (2.0  $\mu$ g/ml). Finally, crystal violet staining cell was used as a measure of cell viability.



**Fig. 2.** Inhibition of TNF/CHX-mediated caspase-3 activation by Antp. HeLa cells were incubated with medium containing increasing concentrations of Antp for 30 minutes at 37°C. Then cells were washed and apoptosis was induced by stimulation with TNF-R1-specific mutant TNF (100 ng/ml) and CHX (2 µg/ml). (A) For measurements of caspase-3 activity cells were harvested 6 hours after the induction of apoptosis. The error bars represent the standard deviation of the mean of three independent experiments. (B) Determination of cell viability was performed 24 hours after TNF/CHX treatment using crystal violet staining.



### Cell-penetrating peptides Antp and R9 inhibit TNF-R1-mediated signalling

To test the hypothesis that the Antp import peptide (Table 1, entry 5) interferes with the TNF-dependent induction of apoptosis, the caspase-3 activity assay was performed in cell lysates from cells pre-treated with the Antp peptide alone. Pre-incubation of cells with Antp significantly reduced the TNF/CHX-induced caspase-3 activity (Fig. 2A) and protected cells against TNF/CHX-induced apoptosis (Fig. 2B) in a dose-dependent manner. To exclude the possibility that the inhibitory effect of Antp was due to the carboxyfluorescein moiety of the peptide we performed the same experiment with unlabelled Antp and observed the same inhibitory effect on the caspase-3 activity (data not shown).

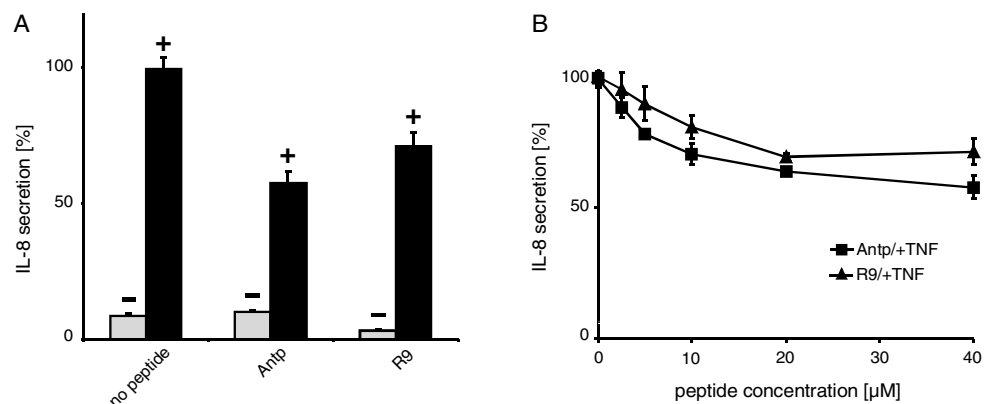
Knowing that TNF-R1 stimulation in the absence of CHX leads to gene induction, we wanted to clarify whether this response was also affected by CPPs. The effect of Antp and nona-arginine (Table 1, entry 6) on gene induction was analysed by measuring TNF-induced IL-8 secretion. Both peptides significantly inhibited the TNF-induced IL-8 secretion in a dose-dependent manner (Fig. 3A,B). At a concentration of 5 µM the Antp peptide already caused a reduction of IL-8 secretion by about 20%. To exclude the possibility that the reduction of cytokine production was due to cytotoxic effects of the peptides, the toxicity of both peptides was tested using crystal violet staining and the MTT assay. The crystal violet assay detects

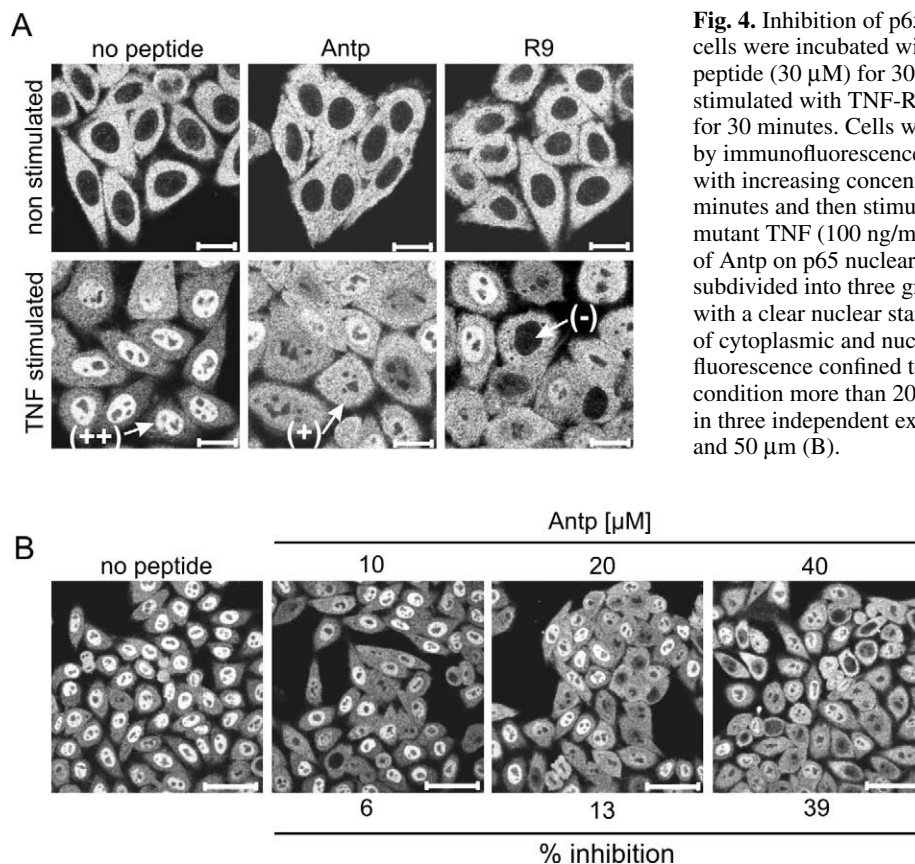
cytotoxicity resulting in a reduction of cell number and MTT also detects an impairment of the metabolic activity of cells. Using both assays no toxicity was observed for concentrations of up to 40 µM for both peptides (data not shown).

### Cationic CPPs inhibit the nuclear translocation of p65

The synthesis of cytokines in response to receptor stimulation is the result of a signalling cascade culminating in the activation and nuclear translocation of the transcription factor NF-κB. Therefore we next investigated whether Antp and R9 interfere with the NF-κB activation cascade. The cellular localization of the NF-κB p65 subunit was visualized by immunofluorescence. In untreated resting cells p65 was only found in the cytoplasm (Fig. 4A). Stimulation of HeLa cells with TNF resulted in a pronounced translocation of p65 from the cytoplasm into the nucleus. Pre-incubation of cells with either Antp or R9 for 30 minutes led to a significant inhibition of this translocation of p65 (Fig. 4A). Incubation with CPPs alone had no effect on p65 localization. Since the inhibition of IL-8 secretion was dependent on peptide concentration, we next examined whether the reduction of p65 nuclear translocation exhibited a similar concentration dependence. Incubation of HeLa cells with different concentrations of Antp prior to TNF stimulation revealed that this was indeed the case (Fig. 4B).

**Fig. 3.** Inhibition of IL-8 induction by cell-penetrating peptides. (A) HeLa cells were incubated with medium containing the indicated peptides (40 µM) for 30 minutes at 37°C. After washing, cells were stimulated with TNF-R1-specific mutant TNF (100 ng/ml) for 5 hours (black bars) or left untreated (grey bars). IL-8 present in the supernatants as determined by ELISA was normalized on the cell number determined by MTT assay. (B) Cells were incubated with increasing concentrations of Antp or R9 for 30 minutes, stimulated with TNF-R1-specific mutant TNF (100 ng/ml) for 5 hours and IL-8 expression was determined. The error bars represent the standard deviation of the mean of three samples.





**Fig. 4.** Inhibition of p65 nuclear translocation. (A) HeLa cells were incubated with medium containing the indicated peptide (30 μM) for 30 minutes, washed and then stimulated with TNF-R1-specific mutant TNF (100 ng/ml) for 30 minutes. Cells were then fixed and p65 was detected by immunofluorescence. (B) HeLa cells were incubated with increasing concentrations of Antp peptide for 30 minutes and then stimulated with the TNF-R1-specific mutant TNF (100 ng/ml). To quantify the inhibitory effect of Antp on p65 nuclear translocation, cells were subdivided into three groups and counted manually: cells with a clear nuclear staining (++), cells with equal levels of cytoplasmic and nuclear staining (+) and cells with fluorescence confined to the cytoplasm (-). For each condition more than 200 cells were counted and analysed in three independent experiments. Scale bars: 20 μm (A) and 50 μm (B).

In contrast to TNF-mediated NF-κB activation that occurs within 15–30 minutes, induction of apoptosis by TNF-R1 requires several hours of TNF treatment (Fotin-Mleczek et al., 2002). Nevertheless, both responses were inhibited when cells were pre-incubated with the peptide for 30 minutes, indicating that (i) the inhibitory effect persisted over several hours after removal of peptide and (ii) CPPs interfere with an early stage of the TNF signalling cascade, upstream of the signal divergence to either NF-κB activation or induction of apoptosis.

#### A cytoplasmic localization of CPPs is not required for the peptide-mediated inhibition of p65 nuclear translocation

To narrow the molecular target on which cationic CPPs exert the observed effects, we next analysed whether cytoplasmic localization of these peptides is required for their action. For this purpose the effect of the CPPs on the TNF-dependent nuclear translocation of NF-κB was detected in the presence of bafilomycin A1 (Baf A1), an inhibitor of endosomal acidification that abolishes the release of cationic CPPs from the endosomal compartment (Fischer et al., 2004). When HeLa cells were treated with Baf A1, fluorescence was exclusively vesicular at any concentration of peptide tested (Fig. 5A). However, pre-incubation of these cells with cationic CPPs still inhibited TNF-mediated NF-κB translocation (Fig. 5B). In control cells, stimulated only with TNF, Baf A1 had no effect on the translocation of NF-κB. We therefore concluded that the release of the peptides into the cytoplasm was not required for the inhibition of TNF signalling. Taken together our results

show that the inhibitory activity of cationic CPPs is not based on an interaction with a cytoplasmic target molecule.

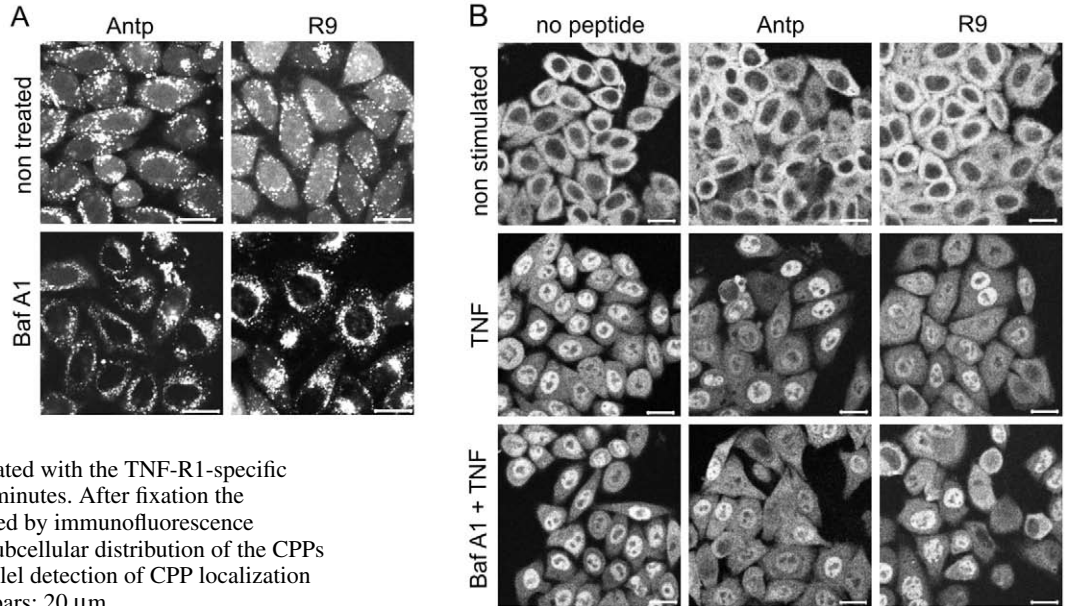
#### CPPs induce the internalization of TNF receptors

Having excluded that the CPPs inhibit TNF-dependent signalling by interfering with molecular events in the cytoplasm we next asked whether the CPPs exerted their effect by impairing the accessibility of the receptor for its ligand on the cell surface. Endocytosis is critically involved in the cellular delivery of CPPs as well as in the signal transduction mediated by different receptors. We therefore investigated whether the endocytosis-dependent uptake of cationic CPPs interfered with the localization of TNF-R1 on the plasma membrane. Cells were incubated with Antp, R9 or Tat (Table 1, entry 7) and the level of TNF-R1 present on the cell surface was quantified using flow cytometry. In addition to Antp and R9, the HIV-1 Tat-derived peptide was also included in this experiment, because this cationic peptide has been used as a CPP in a number of applications for the delivery of DNA and peptides into cells (Eguchi et al., 2001; Choi et al., 2003). All three peptides induced a concentration-dependent downregulation of the receptor (Fig. 6A). The strongest effect was obtained with Antp. HeLa cells incubated with a medium containing Antp at a concentration of 20 μM expressed only 60% of TNF-R1 on the plasma membrane that was observed for untreated cells. The downregulation of TNF-R1 caused by Tat was weaker but nevertheless this CPP also led to a 25% reduction of the receptors on the cell surface. Downregulation of receptors at the plasma membrane can occur either by the



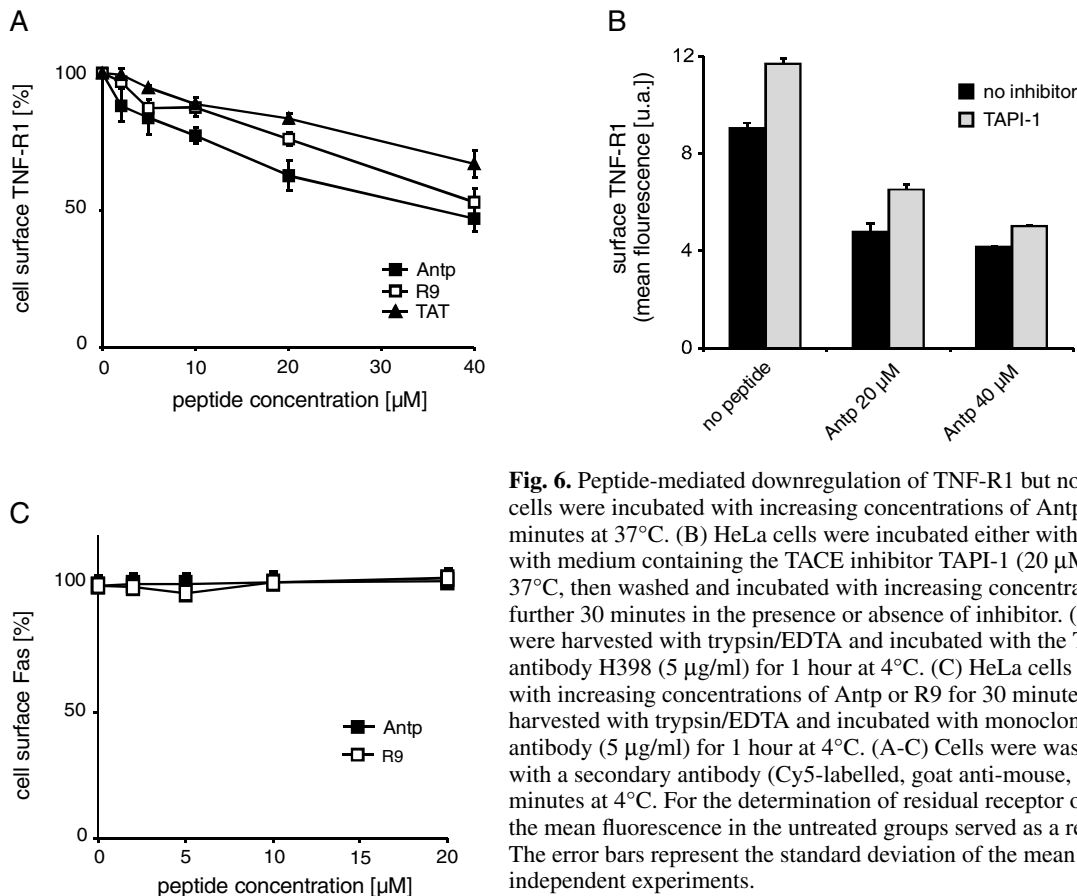
**Fig. 5.** Influence of bafilomycin A1 on the cellular localization of CPPs and on the nuclear translocation of p65.

(A) HeLa cells were treated with Baf A1 (300 nM) for 30 minutes, washed and incubated with Antp or R9 (each 30  $\mu$ M) for a further 30 minutes at 37°C before being examined by confocal microscopy at RT. (B) In a second experiment, after incubation with Baf A1 and peptide as above, cells were washed with medium and stimulated with the TNF-R1-specific mutant TNF (100 ng/ml) for 30 minutes. After fixation the distribution of p65 was determined by immunofluorescence microscopy. The change of the subcellular distribution of the CPPs upon fixation precluded the parallel detection of CPP localization and NF- $\kappa$ B translocation. Scale bars: 20  $\mu$ m.



internalization of whole receptor molecules or by shedding of their extracellular domains (Porteu and Nathan, 1990). To clarify which mechanism was responsible for the downregulation of TNF-R1 by CPPs, we incubated cells with Antp in the presence of a TACE (TNF $\alpha$  converting enzyme) inhibitor (Mullberg et al., 1995). TACE is the

metalloproteinase that processes membrane bound TNF as well as TNF receptors to the respective soluble molecules (Newton et al., 2001). Nevertheless, the incubation of cells with Antp induced a reduction of cell surface receptor molecules by about 50%, independent of the presence of the TACE inhibitor (Fig. 6B). This result strongly suggests that



**Fig. 6.** Peptide-mediated downregulation of TNF-R1 but not Fas. (A) HeLa cells were incubated with increasing concentrations of Antp, R9 and Tat for 30 minutes at 37°C. (B) HeLa cells were incubated either with medium alone or with medium containing the TACE inhibitor TAPI-1 (20  $\mu$ M) for 1 hour at 37°C, then washed and incubated with increasing concentrations of Antp for a further 30 minutes in the presence or absence of inhibitor. (A and B) Cells were harvested with trypsin/EDTA and incubated with the TNF-R1-specific antibody H398 (5  $\mu$ g/ml) for 1 hour at 4°C. (C) HeLa cells were incubated with increasing concentrations of Antp or R9 for 30 minutes at 37°C, washed, harvested with trypsin/EDTA and incubated with monoclonal anti-CD95 (Fas) antibody (5  $\mu$ g/ml) for 1 hour at 4°C. (A-C) Cells were washed and incubated with a secondary antibody (Cy5-labelled, goat anti-mouse, 1:500) for 30 minutes at 4°C. For the determination of residual receptor on the cell surface the mean fluorescence in the untreated groups served as a reference (100%). The error bars represent the standard deviation of the mean of three independent experiments.

internalization of TNF-R1 rather than the induction of shedding is responsible for Antp-mediated downregulation. Next we asked whether the induction of receptor internalization could be observed for other members of the TNF receptor family. First, the death receptor Fas was probed for induction of internalization by CPPs (Fig. 6C). However, in contrast to TNF-R1, incubation with CPPs was without effect on Fas on the cell surface.

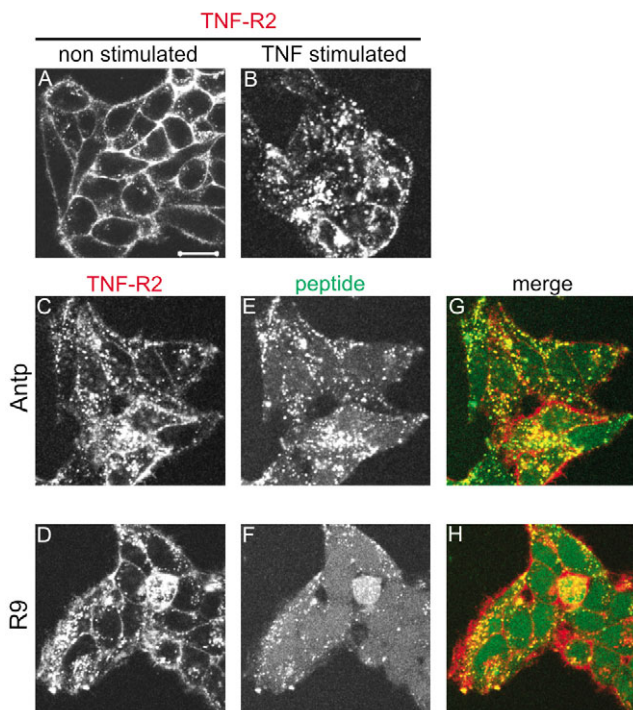
Next, we determined the effect of the cationic CPPs on TNF-R2, which in contrast to TNF-R1 and Fas does not contain a so called death domain and does not induce apoptosis. Because HeLa cells do not express detectable amounts of TNF-R2 a HeLa cell line stably overexpressing TNF receptor 2 (HeLa-TNF-R2) was selected as the experimental system. For TNF-R2, as well, incubation with Antp, R9 or Tat peptide induced a strong downregulation (data not shown). Furthermore, for HeLa-TNF-R2 cells stimulated with a TNF-R2-specific mutant TNF, preincubation with Antp and R9 also led to a reduction in IL-8 secretion (data not shown).

In contrast to the endogenously expressed TNF-R1 the expression level of the TNF-R2 in these cells enabled the analysis of receptor distribution in response to peptide treatment using fluorescence microscopy. The receptors were visualized by immunofluorescence in living cells. In order to avoid receptor cross-linking during incubation with antibodies, a primary non-activating anti-TNF-R2 antibody was incubated

with Alexa Fluor 647-labelled Fab fragments, which bind to the Fc part of IgG. This immune complex itself does not induce receptor internalization. In non stimulated cells, TNF-R2 immunofluorescence was nearly continuous over the plasma membrane (Fig. 7A). TNF stimulation of cells resulted in the clustering of fluorescence and subsequent internalization of receptors (Fig. 7B). The incubation of cells with either Antp or R9 resulted in uptake of the peptides (Fig. 7E,F) and in a clear internalization of TNF-R2 (Fig. 7C,D). Both, peptides and immunostained receptors co-localized within vesicular structures (Fig. 7G,H). To investigate whether the internalization of these receptors depended on the cell type we performed similar experiments with myelomonocytic THP-1 cells, which express a higher level of endogenous TNF-R2. In this cell line Antp also promoted a strong TNF-R2 internalization (data not shown).

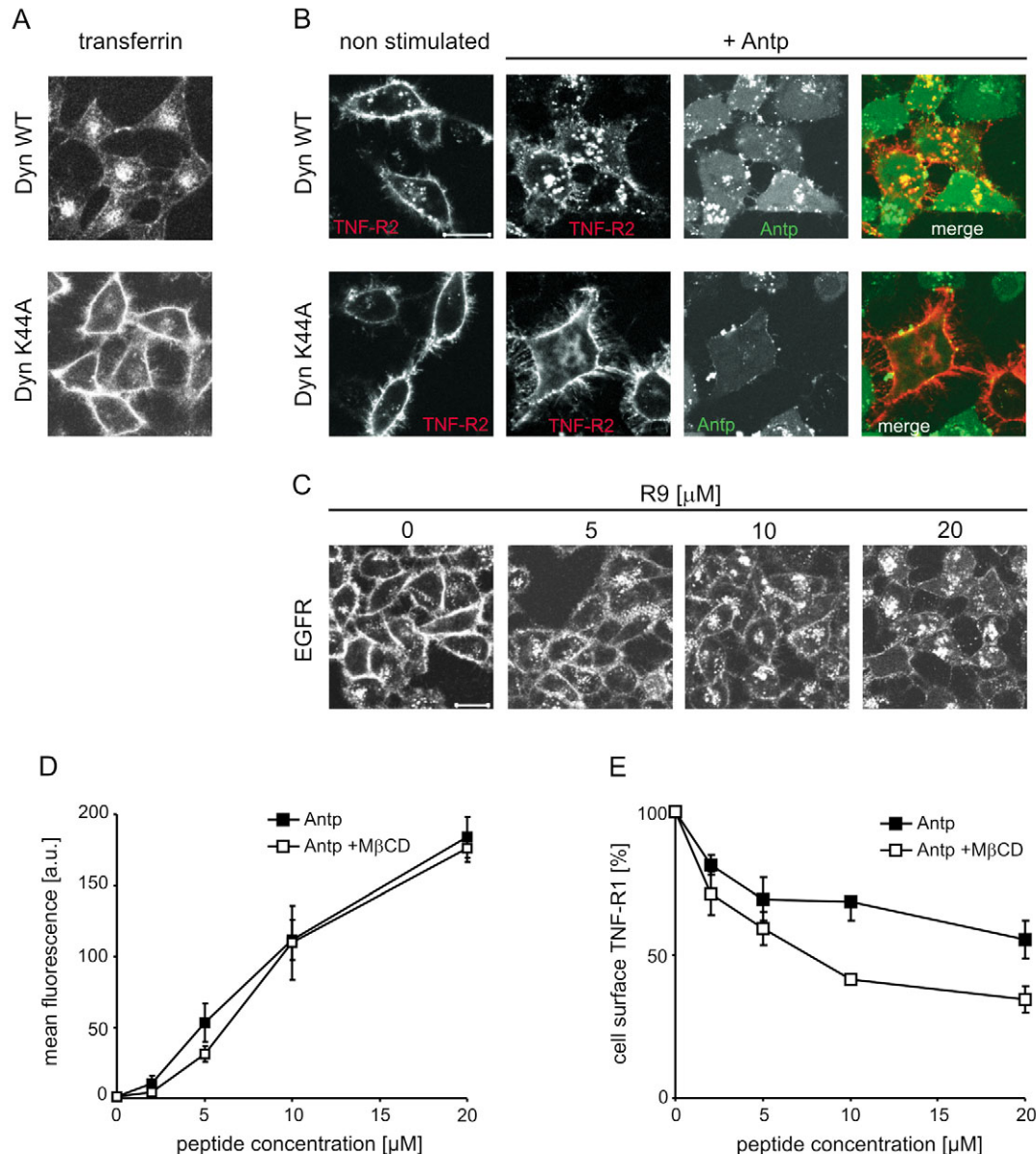
#### Cationic CPPs mediate receptor internalization through clathrin-coated vesicles

Next, we investigated the molecular mechanism by which cationic peptides induce the internalization of TNF receptors. Normally, molecular events leading to the activation of the non-constitutive endocytic machinery are triggered by receptor activation. A protein critically involved in receptor-mediated endocytosis is dynamin. In order to address the possible involvement of this protein in peptide uptake itself and in peptide-induced receptor internalization, HeLadynK44A cells expressing a dominant negative (DN) form of dynamin-2 controlled by a tet off promoter were employed. In the presence of tetracycline, these cells express the wild-type (WT) form of dynamin. In this way, the activation of compensatory mechanisms is avoided. The presence of DN mutant dynamin upon tetracycline deprivation was verified using a fluorescent transferrin as a marker for dynamin-dependent endocytosis. Tetracycline deprivation for 48 hours resulted in the inhibition of transferrin internalization (Fig. 8A). To enable the analysis of dynamin-dependent receptor internalization by immunofluorescence microscopy, HeLadynK44A were transfected by electroporation with a plasmid coding for human TNF-R2 (Fig. 8B). Incubation of HeLadynK44A cells expressing WT dynamin in medium containing Antp led to peptide uptake and to the internalization of TNF-R2 (Fig. 8B, upper panels). As before, receptors and peptide strongly co-localized within vesicles. In contrast, expression of the DN form of dynamin resulted in the almost complete inhibition of peptide uptake and receptor internalization (Fig. 8B, bottom panels). To obtain more detailed information on the endocytic mechanism involved in CPP-induced receptor internalization we specifically tested the involvement of clathrin-coated pits in this process. For this purpose the effect of the cationic CPP on human EGFR (epidermal growth factor receptor), which is internalized via clathrin-mediated endocytosis (Vieira et al., 1996; Jiang et al., 2003) was investigated. R9 induced a concentration-dependent internalization of EGFR (Fig. 8C). Being aware that dynamin is also required for lipid raft-mediated endocytosis we finally addressed whether disruption of these membrane microdomains by cholesterol extraction inhibited peptide uptake and receptor internalization. The inhibition of lipid raft-mediated



**Fig. 7.** Peptide-mediated internalization of TNF-R2. After immunostaining of TNF-R2 with TNF-R2-specific antibody/Zenon Alexa Fluor 647 conjugates, HeLa-TNF-R2 cells were washed and left unstimulated (A), stimulated with TNF-R2-specific mutant TNF (100 ng/ml) for 30 minutes at 37°C (B) or incubated with Antp or R9 (each 5 µM) for 1 hour at 37°C (C,D). Cells were analysed by multichannel confocal microscopy: (A–D) receptor staining; (E,F) distribution of fluorescent peptides; (G,H) superposition of both channels. Scale bar: 20 µm.



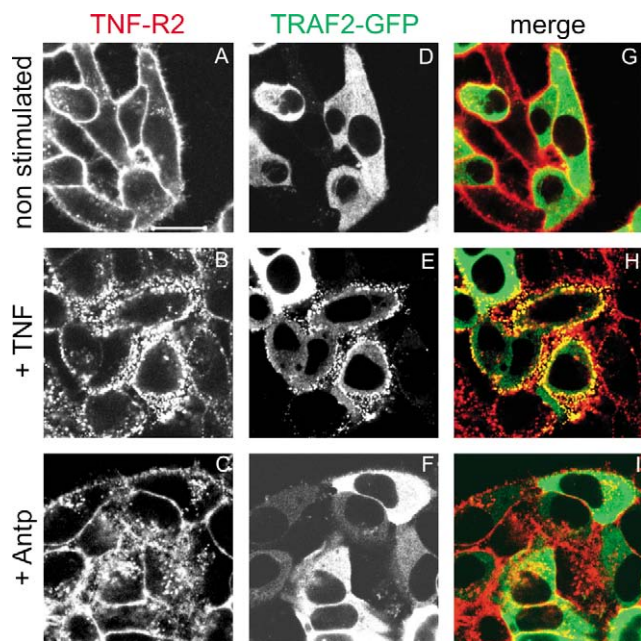


**Fig. 8.** CPP mediate internalization of receptors in a dynamin-dependent but lipid raft-independent manner. (A) HeLadynK44A cells were incubated with or without tetracycline for 48 hours at 37°C, washed and then incubated with medium containing 20 μg/ml Alexa Fluor 633-labelled transferrin for 30 minutes at 37°C. After washing, cells were analysed by confocal microscopy. (B) HeLadynK44A cells were transfected by electroporation with a plasmid coding for human TNF-R2 and incubated for 48 hours at 37°C either with or without tetracycline. TNF-R2 was labelled with a TNF-R2-specific antibody/Zenon Alexa Fluor 647 conjugate. Cells were incubated with Antp (20 μM) for 30 minutes at 37°C, or remained untreated. After washing with medium cells were analysed by multichannel confocal microscopy. (C) EGFR was labelled with Zenon Alexa Fluor 647 conjugate for 30 minutes at 4°C, then cells were washed and incubated with R9 peptide for 30 minutes at 37°C. Scale bar: 20 μm. (D,E) HeLa cells were treated with methyl-β-cyclodextrin (MβCD; 5 mM) for 30 minutes then incubated with Antp in the presence or absence of the inhibitor for further 30 minutes. Cells were then harvested with trypsin/EDTA, TNF-R1 was immunostained and cells were analysed by flow cytometry. Peptide-associated fluorescence (fluorescein) and Alexa Fluor 647 signal were detected simultaneously.

endocytosis through the treatment of cells with methyl-β-cyclodextrin (MβCD) had no effect on the uptake of Antp (Fig. 8D). Also, Antp-mediated TNF-R1 internalization was not inhibited. Instead, a further increase in receptor internalization was observed (Fig. 8E). We, therefore, concluded that CPP-mediated internalization of receptors involves clathrin-coated vesicles and that this process is not restricted to the TNF receptors.

#### The CPP-mediated internalization of TNF-R2 is decoupled from the signal transduction

It has been reported for a number of receptors that activation leads to the initiation of dynamin-dependent endocytosis and thereby downregulation of activated receptors from the plasma membrane (Mitchell et al., 2004). However, pre-incubation of cells with cationic CPPs led to the inhibition of TNF-mediated responses (Figs 2-5) indicating that internalization occurred in



**Fig. 9.** Antp-mediated internalization of TNF-R2 occurs without recruitment of TRAF2. HeLa-TNF-R2 cells were transfected by electroporation with a plasmid coding for a TRAF2-GFP fusion protein. After 24 hours TNF-R2 was stained using a TNF-R2-specific antibody/Zenon Alexa Fluor 647 conjugate. After washing, cells were either stimulated with TNF-R2-specific mutant TNF (300 ng/ml, 30 minutes at 37°C) or incubated with unlabelled Antp (20 μM, 30 minutes at 37°C) or left untreated: (A–C) receptor staining; (D–F) TRAF2-GFP; (G–I) superposition of both channels. Scale bar: 20 μm.

the absence of receptor activation. It cannot be ruled out that incubation of cells with CPPs induces early signalling events, leading to receptor activation and internalization, but fails to activate downstream events required for cytokine expression. The first event in TNF-R2-dependent signal transduction is the binding of the signalling protein TRAF2 (TNF receptor-associated factor 2) to the receptor and the subsequent internalization of ligand/receptor/TRAF2 complexes. Recruitment of TRAF2 to the TNF-R2 is therefore a reliable indicator for receptor activation. In order to enable the analysis in living cells, HeLa-TNF-R2 cells were transfected with a plasmid coding for a TRAF2-GFP fusion protein. In non stimulated cells TRAF2 was localized in the cytoplasm. In response to TNF the fusion protein co-localized with the receptor within vesicular compartments (Fig. 9, middle panels). In contrast Antp-mediated internalization of TNF-R2 had no effect on the cellular distribution of TRAF2 (Fig. 9, bottom panels). No recruitment of the TRAF2 fusion protein to the internalized receptor was observed. This observation, together with the IL-8 and caspase-3 data, shows that cationic CPPs reduce the TNF sensitivity of cells by the downregulation of both TNF receptors.

#### The HIV-1 Tat protein induces the internalization of TNF-R2

The cationic CPPs were developed as molecular tools to promote the cellular uptake of molecules. Both Antp and Tat

were identified as the protein transduction domains of proteins, that enter cells and modulate gene expression. We therefore asked whether the induction of receptor internalization could also be observed for the full length Tat protein. If so, this finding might have implications for the pathophysiological relevance of this protein. HeLa-TNF-R2 cells were incubated with a fluorescein-labelled Tat protein (Fig. 10). In non treated cells only limited internalization of immunostained TNF-R2 molecules was detected (left panel). In contrast, in cells incubated with the Tat protein a pronounced internalization of TNF-R2 was observed. Both, the Tat protein and TNF-R2 co-localized within the same endocytic vesicles (right panel). Tat protein-induced receptor internalization was observed with as little as 0.5 μM Tat protein. Again, no redistribution of TRAF2 was observed demonstrating that the internalization was again independent of receptor activation (data not shown).

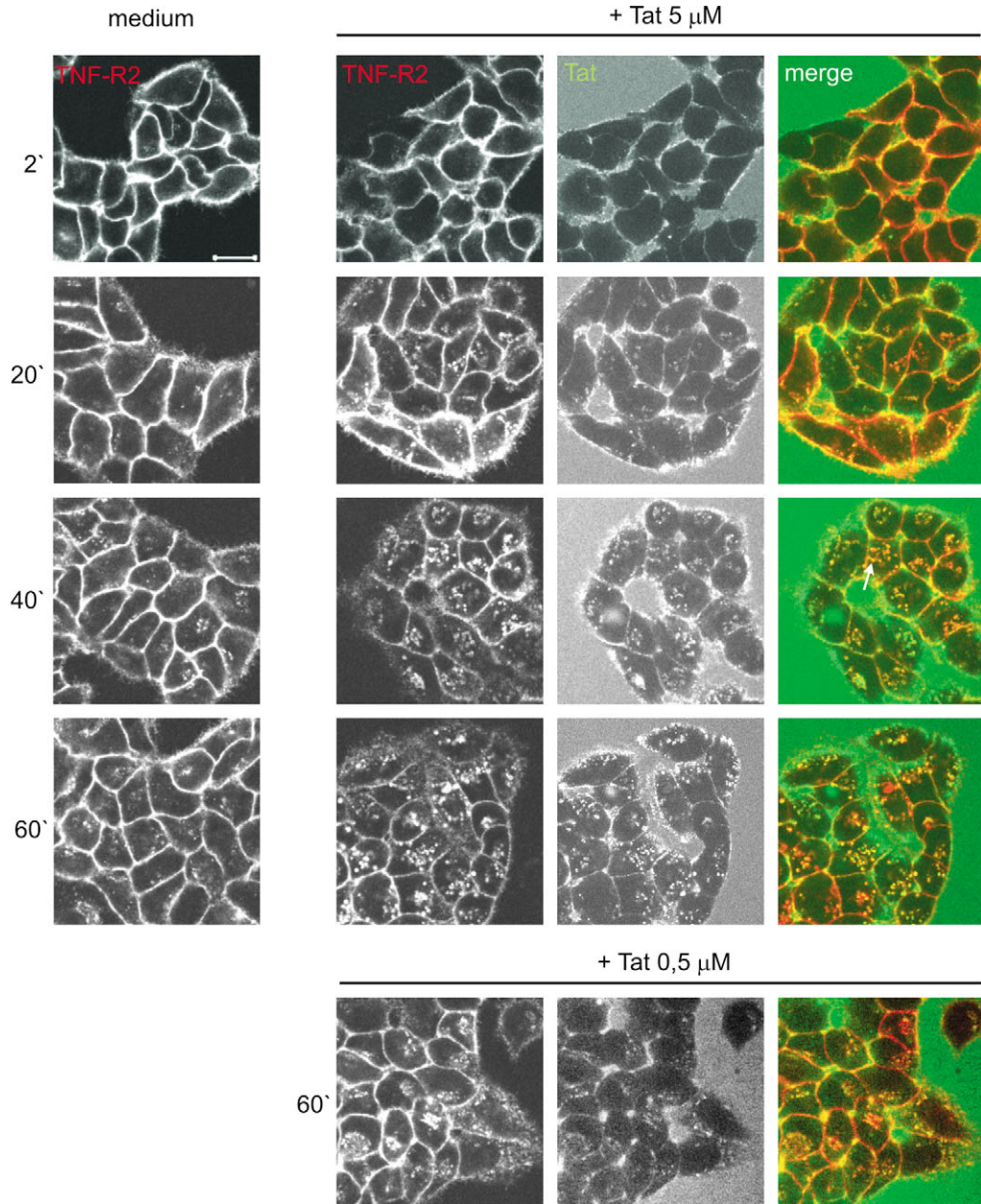
#### Discussion

The general motion for the use of cell-penetrating peptides is their ability to mediate the non-invasive import of bioactive cargoes into cells. In order to be able to interpret the experimental data obtained in such experiments with respect to the imported cargo, it is necessary (i) that the conjugation of CPP to the cargo does not affect the activity of the cargo, and (ii) that CPPs themselves have no effect on cellular signal transduction. In this context our data reveal new and critical information for the application of cationic CPPs as transfer vehicles.

The discovery of the CPP-dependent inhibition of TNF signalling presented here benefited from the fact that a cargo peptide with a biological activity opposing that of Antp was selected. While the Smac peptide enhanced TNF/CHX-mediated apoptosis, Antp inhibited TNF-dependent signal transduction. Smac peptides themselves do not induce any caspase activation directly, but only eliminate the inhibitory action of IAPs on the caspases, which are activated in response to an apoptotic stimulus (Chai et al., 2000). Therefore, the induction of apoptosis is an essential prerequisite for Smac peptides to execute their function. At concentrations lower than 40 μM the pro-apoptotic effect of the Smac peptide remained undetectable and the effect of Antp prevailed. Only the highest peptide concentration produced a clear shift from protection against TNF/CHX-mediated apoptosis to the promotion of an apoptotic response. In contrast, the control peptide Rev-Antp, lacking the bioactive pro-apoptotic sequence but still containing the Antp moiety, almost completely abolished TNF/CHX-induced caspase-3 activation. The results obtained for the Smac-Antp and Rev-Antp peptides at a concentration of 40 μM support the specific pro-apoptotic effect of the Smac sequence and exclude a general concentration-dependent toxicity of peptides as a cause for the increasing caspase-3 activation. In addition, our data agree with other reports demonstrating that the Smac-peptide coupled to the Antp and oligoarginine CPPs, and used at a concentration higher than 25 μM, enhanced apoptosis (Fulda et al., 2002; Arnt et al., 2002). However, there had not been any previous reports of the antagonistic effects of these peptides at lower concentrations.

In comparison to the CPP-mediated uptake, the direct transfer of the Smac peptide via electroporation resulted in a strong enhancement of TNF/CHX-mediated caspase-3 activity





**Fig. 10.** Tat protein induces internalization of TNF-R2. After immunostaining of TNF-R2 with a TNF-R2-specific antibody/Zenon Alexa Fluor 647 conjugate, HeLa-TNF-R2 cells were washed and incubated with fluorescein-labelled Tat protein. Cells were analysed by multichannel confocal microscopy; the first images were taken immediately (2 minutes) after protein addition and further images were taken as indicated. Scale bar: 20  $\mu$ m.

even at lower peptide concentrations. Our analyses suggest that the high bioactivity of the electroporated Smac peptide in comparison to Smac-Antp was the result of two additive effects: first, electroporation of the Smac peptide did not cause internalization of TNF receptors and therefore allowed the efficient induction of apoptosis via TNF-R1, and second, electroporation mediates a direct transfer of peptides into the cytoplasm (Gehl, 2003) favouring the direct interaction of the peptides with their cytoplasmic target proteins. These results confirm that release of intact peptides from the endosomal compartment into the cytoplasm is indeed a limiting step in the CPP-mediated delivery of peptides.

Interestingly, TNF-R1, TNF-R2 and Fas, belonging to the same family of TNF receptors, but exerting different biological effects, behaved differently upon incubation of cells with CPPs. Although TNF-R1 and TNF-R2 were internalized after

incubation with CPPs, Fas remained unaffected. These differences may relate to the association of Fas and the TNF receptors with distinct membrane microdomains. Membrane microdomains, defined by a particular composition of lipids and proteins, promote the segregation of membrane proteins and the generation of pre-assembled signalling complexes within the plasma membrane (Smart et al., 1999).

So far, discrepancies exist concerning the association of the TNF receptors and Fas with specific membrane microdomains. Our data demonstrate that Antp uptake, as well as TNF-R2 internalization, are strongly dynamin dependent. Dynamin is involved in the endocytosis of clathrin-coated vesicles (CCV) (van der Blik et al., 1993) as well as clathrin-independent uptake of caveolae (Henley et al., 1998) and non-caveolar lipid rafts (Lamaze et al., 2001). The CPP-dependent internalization of the EGFR strongly favours clathrin-dependent endocytosis



as the endocytic pathway induced by CPPs. Consistent with a prominent role of clathrin-dependent endocytosis, the disruption of lipid rafts did not inhibit peptide entry. Instead, a further and significant increase in peptide-mediated receptor internalization was observed. Previously, lipid rafts, as well as clathrin-coated pits, had been implicated in the cellular import of Tat-peptide fusion proteins (Fittipaldi et al., 2003; Wadia et al., 2004; Vendeville et al., 2004). One may therefore speculate that disruption of lipid rafts shifts the import of CPPs exclusively to clathrin-dependent endocytosis, thereby further promoting the import of receptors from this microdomain, while maintaining the total rate of Antp uptake constant. Alternatively, in the presence of lipid rafts, a fraction of the receptors may also localize to these membrane microdomains and therefore not be affected by the CPP-dependent induction of internalization.

Even if lipid rafts are left intact, cationic CPPs apparently exert a different effect on these microdomains. For Fas, strong evidence exists that this receptor is localized in lipid rafts (Cremesti et al., 2001; Gajate and Mollinedo, 2001), but no CPP-dependent internalization of this receptor was observed. However, there is a cell-type dependence in the distribution of Fas in the plasma membrane (Muppidi and Siegel, 2004). The CPP-dependent induction of receptor internalization strongly suggests that membrane-associated CPPs are not endocytosed by constitutive membrane turnover. Instead, CPPs themselves promote the internalization of membrane domains. We therefore propose that TNF receptors are localized within membrane microdomains that are predestined for the formation of coated pits.

In contrast to CPP-induced endocytosis, the internalization of receptors in response to TNF begins with the crosslinking of the receptor molecules in the plasma membrane and generation of large receptor clusters (see Fig. 9 middle panels). This ligand-induced receptor clustering represents a critical step in the initiation of TNF-mediated signal transduction (Chan et al., 2000). Very likely, the lack of the receptor aggregation during incubation with the Antp peptide is responsible for the decoupling of receptor internalization from signal transduction.

Surprisingly, the induction of receptor internalization was not restricted to cationic CPPs. The full length HIV-1 Tat protein also induced the internalization of TNF-R2. The HIV-1 Tat protein contributes to the pathophysiology of HIV infections by modulating the expression of several cellular and viral genes (reviewed by Huigen et al., 2004). Remarkably, thirteen years ago it was noticed that Raji cells transfected with the HIV-1 *tat* gene express fewer cell surface TNF receptors than control cells. However, the mechanism of this response remained unexplained. The authors demonstrated that the downregulation of TNF receptors was not due to a decrease in the expression of the gene coding for the receptor or to an increase in receptor occupancy resulting from the induction of the autocrine production of TNF. Our results may now finally provide the mechanistic basis for this observation by demonstrating that the Tat protein-derived peptide, as well as the Tat protein, downregulate receptors as a consequence of their cellular entry.

The results obtained for the Smac-CPP conjugate show, that at least for TNF-dependent signalling, the CPP itself can induce effects that antagonize the effect of the cargo. For

cargoes inhibiting TNF-dependent signal transduction a potentiation of the effect of the cargo through the CPP would be expected. Knowing that CPP-mediated receptor downregulation is not restricted to one receptor and to one cell type, future applications of cationic CPPs to other pathways need to analyse carefully whether the respective targeted pathway exhibits a similar behaviour.

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