

A thorough understanding of the biology of the TNF/TNFR system is a prerequisite to the safe and effective development of anti-TNF therapeutics. In particular, several factors and mechanisms hypothesized to be involved in the side effects elicited by anti-TNF drugs need to be tested (Curtis et al., 2007; Jacobs et al., 2007; Schneeweiss et al., 2007). These include the differential power of the drugs to neutralize TNF bioavailability and the differential inhibition of TNF signaling events. Despite extensive studies on the molecular biology of TNF/TNFR1 signaling (Micheau and Tschopp, 2003) the functions of TNFR2 are poorly understood. There is an increasing need for a comprehensive understanding of TNF/TNFR2 biology, particularly in terms of the development of TNFR-selective drugs.

In this context, we have used a novel phage-display based screening system (Yamamoto et al., 2003; Shibata et al., 2004, 2008) to develop structural mutants of TNF to help clarify the biology of TNF/TNFR2 interactions. These TNF variants, which exert TNFR2-mediated agonistic or antagonistic activity, might be extremely valuable for elucidating structure-activity relationships between TNF and TNFR2. So far, in order to evaluate the bioactivity of TNF through TNFR2, many researchers have used the TNFR2 over-expressing cell lines (Heller et al., 1992; Weiss et al., 1998), such as rat/mouse T hybridomas transfected with human TNFR2 (PC60-hR2) (Vandenabeele et al., 1992). The PC60-hR2 assay is based on granulocyte macrophage colony-stimulating factor (GM-CSF) secretion mediated by TNF/TNFR2 stimuli. The GM-CSF secretion level is quantified by proliferation of GM-CSF-dependent cell lines or by ELISA. However, this two-step assay system is complicated and the screening process is highly laborious. Thus, there are increasing demands for the development of a simple, highly sensitive screening system that is TNFR2-selective.

In the present study, we developed a simple but highly sensitive cell death-based assay system for evaluating TNFR2-mediated activity. We constructed a lentiviral vector expressing a chimeric receptor derived from the extracellular (EC) and transmembrane (TM) domain of human TNFR2 (hTNFR2) and the intracellular (IC) domain of mouse Fas (mFas). Additionally, to eliminate the influence of the endogenous TNFR1, the chimeric receptor was expressed on TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes (Xu et al., 1999). We found that hTNFR2/mFas-expressing preadipocyte (hTNFR2/mFas-PA) showed about 80-times higher sensitivity after treatment with soluble-TNF and over the conventional method. Furthermore, hTNFR2/mFas-PA could detect not only transmembrane TNF- (tmTNF) but also soluble TNF-activity. The technology described herein will be highly useful both as an assay system for various TNF variants *via* TNFR2 and also as a cell-based drug discovery system for TNFR2 agonists/antagonists.

## 2. Materials and methods

### 2.1. Cell culture

TNFR1<sup>-/-</sup>R2<sup>-/-</sup>, TNFR1<sup>-/-</sup>, and wild-type (wt) preadipocytes established from day 16–17 mouse embryos were generously provided by Dr. Hotamisligil (Harvard School of Public Health, Boston MA). Preadipocytes, 293T cells and

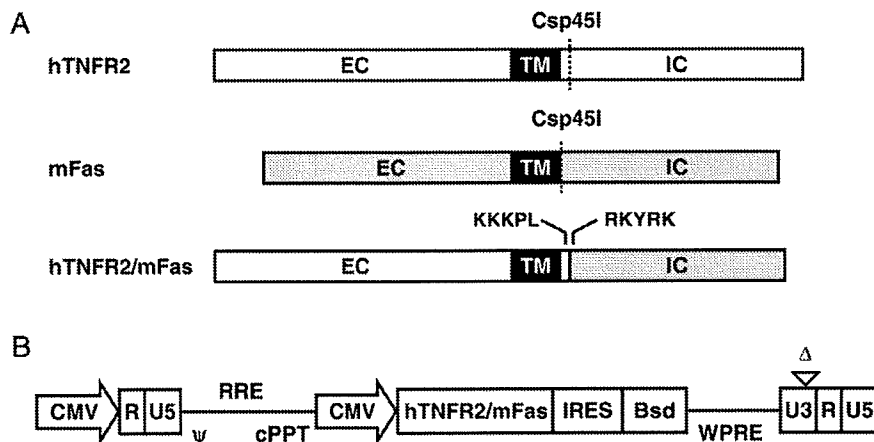
HeLaP4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Inc., Tokyo, Japan) with 10% bovine fetal serum (FBS) and 1% antibiotic cocktail (penicillin 10,000 u/ml, streptomycin 10 mg/ml, and amphotericin B 25 µg/ml; Nacalai Tesque, Kyoto, Japan). The rat/mouse T hybridomas PC60-hR2 cells (hTNFR2 transfected PC60 cells) were generously provided by Dr. Vandenabeele (University of Gent, Belgium) and cultured in RPMI 1640 (Sigma-Aldrich, Inc.) with 10% FBS, 1 mM sodium pyruvate, 5 × 10<sup>-5</sup> M 2-ME, 3 µg/ml puromycin (Wako Pure Chemical Industries, Osaka, Japan), and 1% antibiotic cocktail. TNFR1<sup>-/-</sup>R2<sup>-/-</sup> mouse macrophages were generously provided by Dr. Aggarwal (University of Texas MD Anderson Cancer Center, Houston, TX), and cultured in RPMI 1640 with 10% FBS and 1% antibiotic cocktail.

### 2.2. Construction of self-inactivating (SIN) lentiviral vector

Vectors were constructed using standard cloning procedures. A DNA fragment encoding the EC and TM parts of hTNFR2 was amplified by polymerase chain reaction (PCR) from human peripheral blood lymphocyte cDNA with the following primer pairs: forward primer (5'-GAT TAC GCC AAG CTT GTC GAC CAC CAT GGC GCC CGT CGC CGT CTG GGC CGC GCT GGC CGT CGG ACT GGA G-3') containing a Sall site at the 5'-end and a reverse primer (5'-CAC CTT GGC TTC TCT CTG CTT TCG AAG GGG CTT CTT TTT CAC CTG GGT CA-3') containing a Csp45I site. The resulting amplified fragment was subcloned into pCR-Blunt II-TOPO (Invitrogen Corp., Carlsbad, CA) to generate pCR-Blunt-hTNFR2. A fragment encoding the IC domain of mFas was amplified by PCR from mouse spleen cDNA with the following primer pair: forward primer (5'-AAT TCC ACT TGT ATT TAT ACT TCG AAA GTA CCG GAA AAG A-3') containing a Csp45I site and a reverse primer (5'-GTC ATC CTT GTA GTC TGC GGC CGC TCA CTC CAG ACA TTG TCC TTC ATT TTC ATT TCC A-3') containing a NotI site at the 5'-end. The mFas DNA fragment was subcloned into pCR-Blunt-hTNFR2 between the Csp45I and NotI sites to combine the EC and TM domains of hTNFR2 to the IC domains of mFas, generating pCR-Blunt-hTNFR2/mFas (Fig. 1A). Then the hTNFR2/mFas DNA fragment was cloned between the XhoI and NotI sites of SIN lentiviral vector construct, which contains the blasticidin (Bsd) resistance gene, generating CSII-CMV-hTNFR2/mFas-IRES2-Bsd (Fig. 1B). For construct tmTNF, a DNA fragment encoding non-cleavable human tmTNF h(tmTNFΔ1-12), generated by deleting amino acids 1–12 in the N-terminal part of hTNF, was amplified by PCR from hTNF cDNA with following primer pair: forward primer (5'-AGT GAT CGG CCC CCA GAG GGA AGC TTA GAT CTC TCT CTA ATC AGC CCT CTG GCC CAG GCA GTA GCC CAT GTT GTA GCA AAC CCT CAA G-3') and reverse primer (5'-GGT TGG ATG TTC GTC CTC CGC GGC CGC CTA ACT AGT TCA CAG GGC AAT GAT CCC AAA GTA GAC CTG-3') and cloned into the pY03' vector. Then tmTNFΔ1–12 DNA fragment was cloned between the Sall and XhoI sites of the SIN vector construct, generating CSII-EF-tmTNF-IRES-GFP.

### 2.3. Preparation of lentiviral vectors

The method used to prepare the lentiviral vector has been described previously (Miyoshi et al., 1999; Katayama et al.,



**Fig. 1.** Construction of hTNFR2/mFas chimeric receptor gene and vector. (A) The cDNA structures of hTNFR2, mFas and fusion genes (hTNFR2/mFas) are shown. EC: extracellular domain, TM: transmembrane domain, IC: Intracellular domain. (B) Schematic representation of self-inactivating (SIN) LV plasmid (CSII-CMV-hTNFR2/mFas-IRES-Bsd). CMV, cytomegalovirus promoter;  $\psi$ : packaging signal; RRE, rev responsive element; cPPT, central polypurine tract; IRES, Encephalomyocarditis virus internal ribosomal entry site; Bsd, Blasticidin; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.  $\Delta$ : deleting 133 bp in the U3 region of the 3' long terminal repeat.

2004). In brief, 293T cells were transfected by the calcium phosphate method with three plasmids: packaging construct (pCAG-HIVgp), VSV-G and Rev expressing construct (pCMV-VSV-G-RSV-Rev) and SIN vector construct (CSII-CMV-TNFR2/Fas-IRES2-Bsd or CSII-EF-tmTNF-IRES-GFP). Two days after transfection, the conditioned medium was collected and the virus was concentrated by ultracentrifugation at  $50,000 \times g$  for 2 h at 20 °C. The pelleted virus was resuspended in Hanks' balanced salt solution (GIBCO BRL, Paisley, UK). Vector titers were determined by measuring the infectivity of HeLaP4 cells with serial dilutions of vector stocks using flow cytometric analysis (FCM) for hTNFR2/mFas- or GFP-positive cells.

#### 2.4. Preparation of hTNFR2/mFas- or tmTNF-expressing cell culture

To prepare the hTNFR2/mFas- or tmTNF-expressing cell culture, TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes or TNFR1<sup>-/-</sup>R2<sup>-/-</sup> macrophages were infected with each lentiviral vector at a multiplicity of infection (MOI) of 100. Stable hTNFR2/mFas-transfectants were selected for growth in culture medium containing 8  $\mu\text{g/ml}$  Bsd (Invitrogen Corp.) for 1 week. Expression of hTNFR2/mFas chimeric receptor on Bsd-resistant cells was detected by staining with biotinylated anti-hTNFR2 antibody (BD Biosciences, Franklin Lakes, NJ) at 0.5  $\mu\text{g}/5 \times 10^5$  cells for 30 min at 4 °C. Subsequently, the cells were washed and stained with streptavidin-PE conjugate (BD Biosciences). The cell suspension was centrifuged at  $800 \times g$ , washed with PBS, centrifuged again, and then re-suspended in 500  $\mu\text{l}$  of 0.4% paraformaldehyde. Fluorescence was analyzed on a FACS Vantage flow cytometer, and data were analyzed using CellQuest software (both BD Biosciences). The hTNFR2/mFas-positive cell cultures were used in subsequent experiments as hTNFR2/mFas-PA cells. For preparation of tmTNF-expressing TNFR1<sup>-/-</sup>R2<sup>-/-</sup> macrophages (tmTNF-M $\phi$ ), IRES-driven GFP positive cells were sorted by FACS Aria (BD Biosciences).

#### 2.5. Cytotoxicity assays

Cells were seeded on 96-well micro titer plates at a density of  $1.5 \times 10^4$  cells/well in culture medium. Serial dilutions of mouse or human TNF (mTNF or hTNF; Peprotech, Rocky Hill, NJ), anti-mFas antibody (clone Jo2; BD Biosciences), or paraformaldehyde-fixed tmTNF-M $\phi$  were prepared with DMEM containing 1  $\mu\text{g/ml}$  cycloheximide, and added to each well. After 48 h incubation, the cell viability was measured by WST-8 assay kit (Nacalai Tesque) according to the manufacturer's instructions. The assay is based on cleavage of the tetrazolium salt WST-8 to formazan by cellular mitochondrial dehydrogenase.

#### 2.6. Induction of GM-CSF secretion on PC60-hR2

$5 \times 10^4$  of PC60-hR2 cells were seeded on a 96 well plate and then exposed to a serial dilution of hTNF in the presence of IL-1 $\beta$  (2 ng/ml). After 24 h incubation, hTNFR2-mediated GM-CSF secretion on PC60-hR2 cells was quantified by ELISA kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

#### 2.7. Immunoprecipitation and western blotting

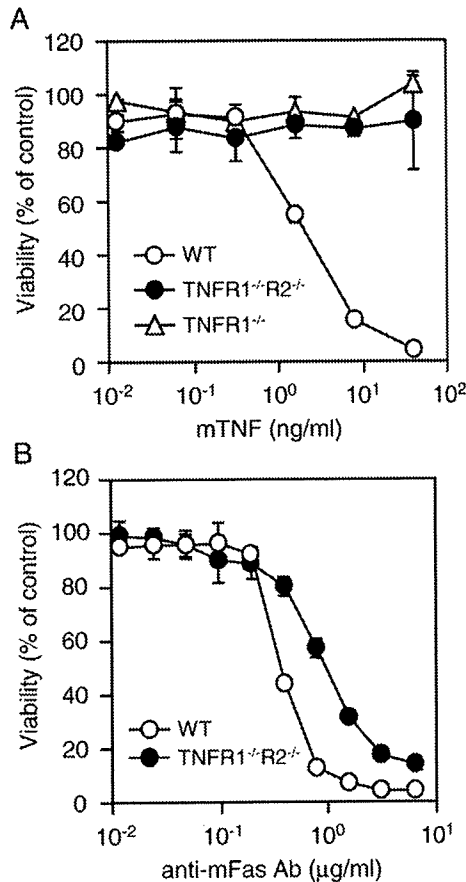
For immunoprecipitation we used FLAG-TNF (a FLAG-tag fusion protein of hTNF), which was generated in *E. coli* and purified in our laboratory. The protocol for the expression and purification of recombinant proteins has been described previously (Yamamoto et al., 2003).  $1 \times 10^7$  hTNFR2/mFas-PA cells were treated with or without 100 ng/ml of FLAG-TNF for 30 min at 37 °C. Cells were then harvested and lysed in 1 ml of lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitor cocktail; Sigma-Aldrich Inc.) and gently rocked at 4 °C for 30 min. Cell debris was removed by centrifugation at  $10,000 \times g$  for 30 min. The resulting supernatant was immunoprecipitated with anti-FLAG-M2 affinity beads (Sigma, St.Louis, MO) for 4 h at 4 °C. Immune complexes bound to the beads were washed three

times with 500  $\mu$ l of lysis buffer and eluted with 3 $\times$ FLAG peptide at a concentration of 150 ng/ml. Collected proteins were resolved on 10–20% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA) by electroblotting. Western blot analyses were performed with biotinylated anti-hTNFR2 antibody (R&D systems) or anti-FADD (Fas-associated death domain protein) antibody (H-181; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Bound primary antibodies were visualized with horseradish peroxidase-conjugated streptavidin or goat-anti-rabbit-IgG (Jackson ImmunoResearch Lab., West Grove, PA) respectively, and ECL plus western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). A LAS 3000 image analyzer (Fujifilm, Tokyo, Japan) was used for the observation of chemiluminescence.

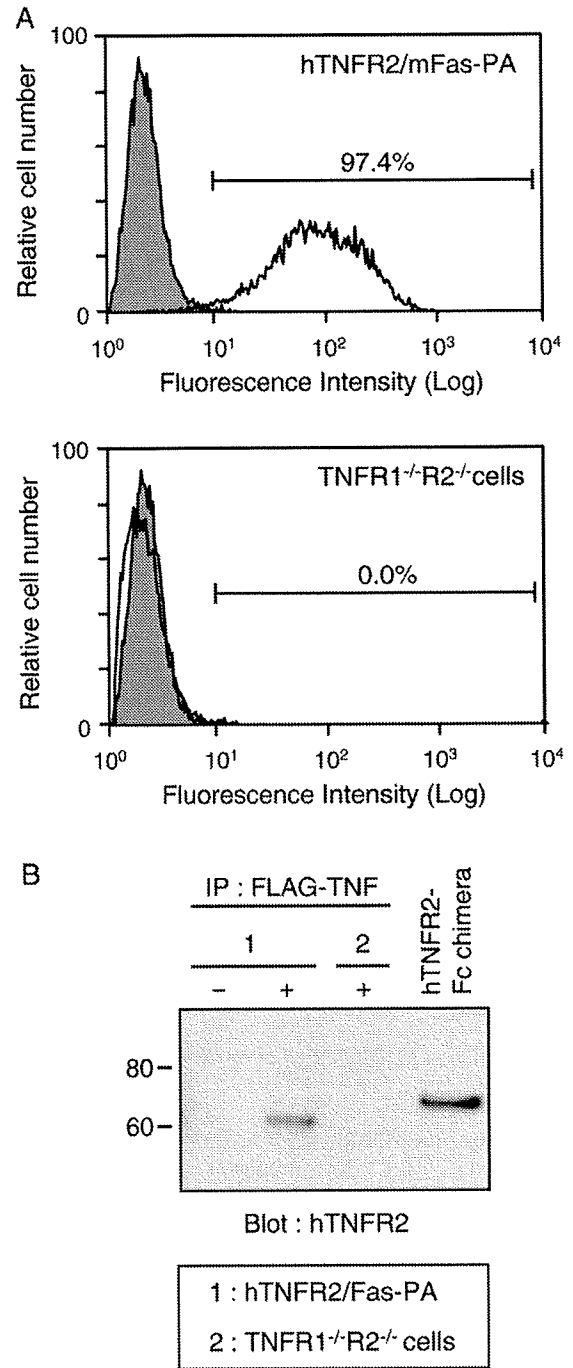
### 3. Results

#### 3.1. Fas- but not TNFR-mediated induction of cell-death in TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes

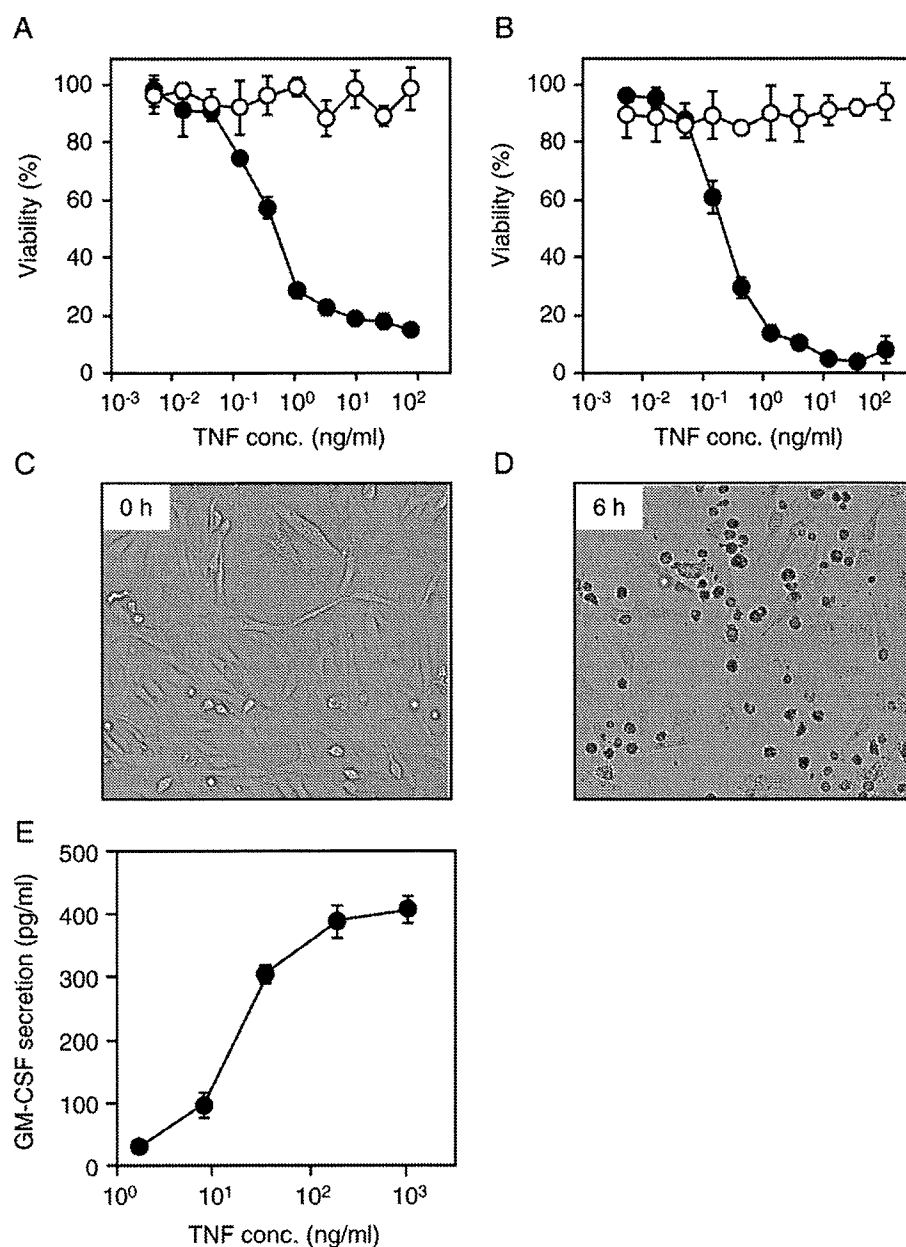
Initially, we established a cell line that could be used to evaluate TNFR2-specific bioactivity by means of the chimeric receptor (hTNFR2/mFas) strategy. The parental cell line must



**Fig. 2.** Fas, but not TNFR2, induced cell death in preadipocytes. WT, TNFR1<sup>-/-</sup>R2<sup>-/-</sup> and TNFR1<sup>-/-</sup> cells were treated with serial dilutions of (A) mTNF or (B) anti-mFas Ab. Cell viability was determined using the WST-8 Assay. Each data point represents the mean  $\pm$  SD of triplicate wells.



**Fig. 3.** Expression of hTNF2/mFas chimeric receptor on transfectants. (A) Expression of the chimeric receptor on hTNFR2/mFas-PA (upper panel) or parental TNFR1<sup>-/-</sup>R2<sup>-/-</sup> cells (lower panel) was analyzed by flow cytometry using hTNFR2-specific antibody (open histograms) or isotype control antibody (shaded histograms). (B) hTNFR2/mFas-PA or TNFR1<sup>-/-</sup>R2<sup>-/-</sup> cells were treated (+) with FLAG-TNF; (-) denotes untreated control cells. Immunoprecipitation was performed with anti-FLAG antibody M2-conjugated beads. After extensive washing, the immunocomplexes were eluted with 3 $\times$ FLAG peptide. Eluted proteins were resolved on 10–20% SDS-PAGE gels and the presence of hTNFR2/mFas in the complex was detected by western blot using anti-hTNFR2 antibody.



**Fig. 4.** Induction of strong cell death on hTNFR2/mFas-PA by soluble hTNF. hTNFR2/mFas-PA (●) and parental TNFR1<sup>-/-</sup>R2<sup>-/-</sup> (○) cells were treated with serial dilutions of hTNF in the presence of cycloheximide (1 μg/ml). After (A) 24 h and (B) 48 h, the cell viability was measured using the WST-8 Assay. Data from the WST-8 assay represents the mean and SDs of triplicate assays. Similar results were obtained in three independent experiments. (C) Untreated or (D) hTNF-treated (10 ng/ml) hTNFR2/mFas-PA cells were incubated for 6 h, and were assessed by light microscopy. (E) PC60-hR2 cells were incubated in the presence of a serial dilution of hTNF and IL-1β (2 ng/ml). After 24 h, induction of GM-CSF was determined by ELISA. Each data point represents the mean ± SD of triplicate measurements.

possess both Fas-sensitivity and TNF-resistance. Thus, we selected TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes as the parental cell line and then examined the susceptibility of this cell line against TNFR1- and Fas-induced cell death. TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes were resistant to TNF-induced cell death, while WT preadipocytes, which co-express both TNFR1 and TNFR2, were killed by mTNF-treatment in a dose-dependent manner (Fig. 2A). TNFR1<sup>-/-</sup> preadipocytes were also resistant

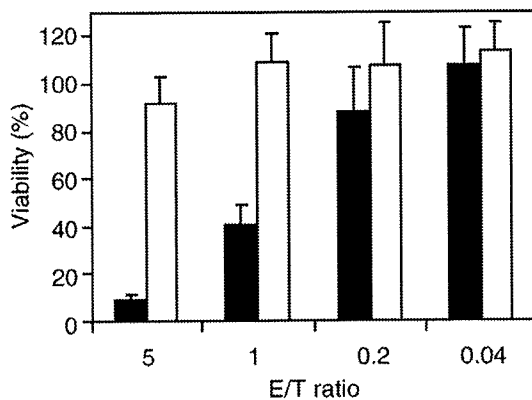
to TNF-induced cell death. Thus, TNF-mediated cell death is presumably due to TNFR1-stimuli in accordance with previous reports (Vandenabeele et al., 1995; Ashkenazi and Dixit, 1998; Devin et al., 2000). Anti-Fas antibody treatment induced cell death for both WT, R1<sup>-/-</sup> and TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes (Fig. 2B). Based on these results, we therefore selected TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes for constructing an hTNFR2/mFas-expressing cell line.

### 3.2. hTNFR2-expression analysis of LV-hTNFR2/mFas-Bsd infected Bsd-resistant cells

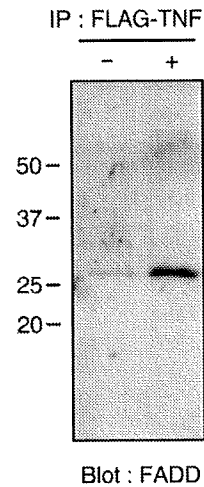
Using the LV technique followed by Bsd selection, we established transfectants that stably expressed hTNFR2/mFas chimeric receptor in which the EC and TM portion of hTNFR2 (amino acids 1–292) was fused to the IC region of mFas (amino acids 187–328) (Figs. 1A and B). FCM analysis revealed that almost 95% of Bsd-resistant cells expressed the EC domain of hTNFR2 (Fig. 3A). To determine whether hTNFR2/mFas retained binding activity against hTNF, we next performed immunoprecipitation and western blot analysis (Fig. 3B). These analyses showed that FLAG-TNFs were immunoprecipitated and eluted with hTNFR2/mFas from LV-transfected and Bsd-resistant cells, but not from parental TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes and untreated cells. Thus, we succeeded in constructing hTNFR2/mFas expressing TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes that retained the ability to bind hTNF.

### 3.3. Induction of apoptosis on hTNFR2/mFas-PA

To examine whether the death signal could be transduced by stimulating the chimeric receptors, we evaluated the cell viability of soluble hTNF-treated hTNFR2/mFas-PA. As anticipated, addition of hTNF to hTNFR2/mFas-PA induced a strong cytotoxic effect 24 and 48 h later, whereas no cell death was detected using parental TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes (Figs. 4A and B). After 48 h, more than 90% of hTNFR2/mFas-PA cells were killed by hTNF at a concentration of 4 ng/ml, resulting in a median effective concentration (EC<sub>50</sub>) of 250 pg/ml. The images in Figs. 4C and D show that hTNFR2/mFas-PA cells underwent clear morphological changes, indicating apoptosis by hTNF stimuli. Additionally, PC60-hR2 cells were tested for hTNFR2-mediated GM-CSF secretion (Fig. 4E). The concentration required to induce 50% of maximal secretion of GM-CSF obtained with hTNF (EC<sub>50</sub>) was approximately 20 ng/ml. Importantly, our bioassay based on cell death phenotype displayed a ~80-fold higher level of sensitivity over conventional methodol-



**Fig. 5.** hTNFR2/mFas-PA cells could be induced cell death by tmTNF. hTNFR2/mFas-PA cells were co-incubated with paraformaldehyde-fixed tmTNF-Mφ (filled bars) or TNFR1<sup>-/-</sup>R2<sup>-/-</sup> Mφ (open bars) at an effector/target (E/T) ratio of 5:1, 1:1, 0.2:1 and 0.4:1 in the presence of cycloheximide (1 μg/ml). After 48 h, cell viability was measured by WST-8 Assay. Each data point represents the mean ± SD of triplicate measurements.



**Fig. 6.** Recruitment of FADD to the hTNFR2/mFas chimeric receptor in response to hTNF. hTNFR2/mFas-PA or TNFR1<sup>-/-</sup>R2<sup>-/-</sup> cells were treated (+) with FLAG-TNF; (-) denotes untreated cells. Immunoprecipitation was performed with anti-FLAG antibody M2-conjugated beads. After extensive washing, immunocomplexes were eluted with 3×FLAG peptide. The eluted proteins were resolved on 10–20% SDS-PAGE gels and the presence of hTNFR2/mFas in the complex was detected by western blot using anti-FADD Antibody.

ogies. Moreover, tmTNF (Fig. 5) and anti-TNFR2 agonistic antibody (data not shown) induced hTNFR2/mFas-PA cell death.

### 3.4. Recruitment of FADD to the hTNFR2/mFas chimeric receptor

Recent studies indicate that some TNFR family members, including Fas, self-associate as trimers prior to ligand binding. Activation of the pre-associated receptors is triggered by ligand-induced rearrangement of the assembled trimers (Algeciras-Schimmich et al., 2002). We speculated that the first reaction after ligand-induced oligomerization of hTNFR2/mFas might be the recruitment of FADD, leading to caspase-8 activation. To investigate the composition of the ligand-hTNFR2/mFas signaling complex, we treated hTNFR2/mFas-PA cells with FLAG-tagged hTNF and affinity purified the receptor complex using anti-FLAG antibody-conjugated beads, followed by western blot analysis with antibody against FADD. As expected, FADD was immunoprecipitated with hTNFR2/mFas on hTNF-treated hTNFR2/mFas-PA (Fig. 6). In similar experiments, we could not detect TRADD, which is recruited to TNFR1 in a ligand-dependent process (data not shown). It has been reported that, in contrast to TNFR1, Fas does not interact with TRADD but directly recruits FADD, leading to efficient cell death (Stanger et al., 1995; Dempsey et al., 2003). Because hTNFR2/mFas interacts with FADD, our hTNFR2/mFas-PA cell-based assay system will be useful for evaluating hTNF activity specifically via hTNFR2.

## 4. Discussion

Here, we developed a hTNFR2/mFas-PA cell-based assay system in order to investigate hTNF activity through hTNFR2. The assay is simple to perform and can detect hTNF-mediated hTNFR2 activity with high sensitivity. Because the hTNFR2/

mFas-PA system was engineered in TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes, we were able to analyze hTNF-mediated hTNFR2 activity without affecting TNFR1-related apoptosis. Moreover, not only tmTNF- but also soluble TNF-mediated hTNFR2 activity was detectable using the hTNFR2/mFas-PA cell system.

In our system, hTNFR2-mediated cytotoxic activity on hTNFR2/mFas-PA cells was quantitatively determined using the WST-8 assay. Alternative methods, such as the MTT assay or Methylene Blue assay, are also capable of detecting cytotoxicity. Using the WST-8 assay, hTNFR2-mediated cytotoxic activity was readily detected 24 h after hTNF treatment (Fig. 4A), although a stronger signal was generated with a longer incubation time (48 h; Fig. 4B). Therefore, 48 h-treatment might be more appropriate for evaluating the activity of a low dose of hTNF or when analyzing an activity-weakened mutant TNF. Furthermore, if you evaluate in the absence of CHX, it may be desirable to alter the cell numbers from 1.5 to 1.0 × 10<sup>4</sup> cells/well. Previously, a similar assay system to the one described in this report was developed by Heidenreich et al. based on murine TNFR1<sup>-/-</sup>R2<sup>-/-</sup> cells, which heterogenetically expressed human Fas conjugated with hTNFR2 (termed MF-R2-Fas cells) (Krippner-Heidenreich et al., 2002). However, there are some important differences between the MF-R2-Fas cells and the cell line used in our assay system (hTNFR2/mFas-PA), such as detachability. Surprisingly, unlike MF-R2-Fas cells, hTNFR2/mFas-PA cells can detect tmTNF-mediated activity as well as soluble TNF activity. Currently, the reason for this difference is unclear, although it may be caused by heterogeneity of the Fas domain. Indeed, the genetic homology between murine Fas and human Fas in IC domain is approximately 50%. Additionally, other factors may account for the observed differences between the two assay systems, such as expression level of the chimeric receptor. At any event, our results suggest that hTNFR2/mFas-PA cells will be useful in providing the basis for a highly sensitive assay system for analyzing hTNFR2 activity mediated by both soluble TNF and tmTNF. We are currently attempting to generate a TNFR2-selective mutant TNF using this assay system and a phage display system for TNF-therapy or anti-TNF therapy (unpublished data). We chose to use hTNFR2/mFas-PA cells in the screening process for a TNFR2-selective mutant TNF because this cell line is sensitive against not only purified TNF but also culture supernatants of TNF-transfected *E. coli* (crude samples). Therefore, our simple and sensitive bioassay enables high-throughput screening for TNFR2-selective mutant TNFs. The TNFR2-selective mutant will make it possible to perform a structure-function study of TNF/TNFR at any stage of function.

With the success of the human genome project, the focus of life science research has shifted to functional and structural analyses of proteins, such as disease proteomics (Oh et al., 2004; Gilchrist et al., 2006). Thus, there is increasing expectation on drug discovery/development based on the information from genomics or proteomics research, structural biology studies, or receptor-ligand interaction analyses. In particular, the therapeutic application of bioactive proteins, such as cytokines and the newly identified ligand proteins, is eagerly awaited (Gollob et al., 2003; Ansell et al., 2006). Surprisingly, however, these ligand proteins display multiple functions, which has severely limited their clinical application

(Margolin et al., 1994; Eskander et al., 1997). Because the reason behind this limitation is that these ligand proteins stimulate different signal transduction pathways via multiple (two or more) receptors, the discovery of receptor-specific agonistic or antagonistic drugs is keenly awaited. Our assay system using a chimeric receptor strategy is applicable to other cytokines and thereby provides a new avenue for identifying receptor-specific agonists or antagonists. We fully anticipate that our novel technology will accelerate the development of TNFR2-related therapeutic molecules as well as acting as a research tool for studying the biology of TNFR2.

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### References

- Aggarwal, B.B., 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat. Rev. Immunol.* 3, 745.
- Algeciras-Schimnich, A., Shen, L., Barnhart, B.C., Murmann, A.E., Burkhardt, J.K., Peter, M.E., 2002. Molecular ordering of the initial signaling events of CD95. *Mol. Cell Biol.* 22, 207.
- Ansell, S.M., Geyer, S.M., Maurer, M.J., Kurtin, P.J., Micallef, J.N., Stella, P., Etzell, P., Novak, A.J., Erlichman, C., Witzig, T.E., 2006. Randomized phase II study of interleukin-12 in combination with rituximab in previously treated non-Hodgkin's lymphoma patients. *Clin. Cancer Res.* 12, 6056.
- Ashkenazi, A., Dixit, V.M., 1998. Death receptors: signaling and modulation. *Science* 281, 1305.
- Brown, S.L., Greene, M.H., Gershon, S.K., Edwards, E.T., Braun, M.M., 2002. Tumor necrosis factor antagonist therapy and lymphoma development: twenty-six cases reported to the Food and Drug Administration. *Arthritis Rheum.* 46, 3151.
- Curtis, J.R., Patkar, N., Xie, A., Martin, C., Allison, J.J., Saag, M., Shatin, D., Saag, K.G., 2007. Risk of serious bacterial infections among rheumatoid arthritis patients exposed to tumor necrosis factor alpha antagonists. *Arthritis Rheum.* 56, 1125.
- Dempsey, P.W., Doyle, S.E., He, J.Q., Cheng, G., 2003. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev.* 14, 193.
- Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M., Liu, Z., 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity* 12, 419.
- Eskander, E.D., Harvey, H.A., Givant, E., Lipton, A., 1997. Phase I study combining tumor necrosis factor with interferon-alpha and interleukin-2. *Am. J. Clin. Oncol.* 20, 511.
- Feldmann, M., Maini, R.N., 2003. Lasker Clinical Medical Research Award. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat. Med.* 9, 1245.
- Gilchrist, A., Au, C.E., Hiding, J., Bell, A.W., Fernandez-Rodriguez, J., Lesimple, S., Nagaya, H., Roy, L., Gosline, S.J., Hallett, M., Paiement, J., Kearney, R.E., Nilsson, T., Bergeron, J.J., 2006. Quantitative proteomics analysis of the secretory pathway. *Cell* 127, 1265.
- Gollob, J.A., Veenstra, K.G., Parker, R.A., Mier, J.W., McDermott, D.F., Clancy, D., Tutin, L., Koon, H., Atkins, M.B., 2003. Phase I trial of concurrent twice-weekly recombinant human interleukin-12 plus low-dose IL-2 in patients with melanoma or renal cell carcinoma. *J. Clin. Oncol.* 21, 2564.
- Heller, R.A., Song, K., Fan, N., Chang, D.J., 1992. The p70 tumor necrosis factor receptor mediates cytotoxicity. *Cell* 70, 47.
- Jacobs, M., Togbe, D., Fremont, C., Samarina, A., Allie, N., Botha, T., Carlos, D., Parida, S.K., Grivennikov, S., Nedospasov, S., Monteiro, A., Le Bert, M.,

- Quesniaux, V., Ryffel, B., 2007. Tumor necrosis factor is critical to control tuberculosis infection. *Microbes Infect.* 9, 623.
- Katayama, K., Wada, K., Miyoshi, H., Ohashi, K., Tachibana, M., Furuki, R., Mizuguchi, H., Hayakawa, T., Nakajima, A., Kadowaki, T., Tsutsumi, Y., Nakagawa, S., Kamisaki, Y., Mayumi, T., 2004. RNA interfering approach for clarifying the PPARGamma pathway using lentiviral vector expressing short hairpin RNA. *FEBS Lett.* 560, 178.
- Krippner-Heidenreich, A., Tubing, F., Bryde, S., Willi, S., Zimmermann, G., Scheurich, P., 2002. Control of receptor-induced signaling complex formation by the kinetics of ligand/receptor interaction. *J. Biol. Chem.* 277, 44155.
- Margolin, K., Aronson, F.R., Sznol, M., Atkins, M.B., Gucalp, R., Fisher, R.I., Sunderland, M., Doroshow, J.H., Ernest, M.L., Mier, J.W., et al., 1994. Phase II studies of recombinant human interleukin-4 in advanced renal cancer and malignant melanoma. *J. Immunother. Emphas. Immunol.* 15, 147.
- Micheau, O., Tschopp, J., 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 114, 181.
- Miyoshi, H., Smith, K.A., Mosier, D.E., Verma, I.M., Torbett, B.E., 1999. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* 283, 682.
- Nathan, D.M., Angus, P.W., Gibson, P.R., 2006. Hepatitis B and C virus infections and anti-tumor necrosis factor-alpha therapy: guidelines for clinical approach. *J. Gastroenterol. Hepatol.* 21, 1366.
- Oh, P., Li, Y., Yu, J., Durr, E., Krasinska, K.M., Carver, L.A., Testa, J.E., Schnitzer, J.E., 2004. Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature* 429, 629.
- Schneeweiss, S., Setoguchi, S., Weinblatt, M.E., Katz, J.N., Avorn, J., Sax, P.E., Levin, R., Solomon, D.H., 2007. Anti-tumor necrosis factor alpha therapy and the risk of serious bacterial infections in elderly patients with rheumatoid arthritis. *Arthritis Rheum.* 56, 1754.
- Shibata, H., Yoshioka, Y., Ikemizu, S., Kobayashi, K., Yamamoto, Y., Mukai, Y., Okamoto, T., Taniai, M., Kawamura, M., Abe, Y., Nakagawa, S., Hayakawa, T., Nagata, S., Yamagata, Y., Mayumi, T., Kamada, H., Tsutsumi, Y., 2004. Functionalization of tumor necrosis factor-alpha using phage display technique and PEGylation improves its antitumor therapeutic window. *Clin. Cancer Res.* 10, 8293.
- Shibata, H., Yoshioka, Y., Ohkawa, A., Minowa, K., Mukai, Y., Abe, Y., Taniai, M., Nomura, T., Kayamuro, H., Nabeshi, H., Sugita, T., Imai, S., Nagano, K., Yoshikawa, T., Fujita, T., Nakagawa, S., Yamamoto, A., Ohta, T., Hayakawa, T., Mayumi, T., Vandenabeele, P., Aggarwal, B.B., Nakamura, T., Yamagata, Y., Tsunoda, S., Kamada, H., Tsutsumi, Y., 2008. Creation and X-ray structure analysis of the tumor necrosis factor receptor-1-selective mutant of a tumor necrosis factor-alpha antagonist. *J. Biol. Chem.* 283, 998.
- Stanger, B.Z., Leder, P., Lee, T.H., Kim, E., Seed, B., 1995. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* 81, 513.
- Szlosarek, P.W., Balkwill, F.R., 2003. Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. *Lancet Oncol.* 4, 565.
- Vandenabeele, P., Declercq, W., Beyaert, R., Fiers, W., 1995. Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol.* 5, 392.
- Vandenabeele, P., Declercq, W., Vercammen, D., Van de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., Fiers, W., 1992. Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T cell hybridoma. *J. Exp. Med.* 176, 1015.
- Wajant, H., Pfizenmaier, K., Scheurich, P., 2003. Tumor necrosis factor signaling. *Cell Death Differ.* 10, 45.
- Weiss, T., Grell, M., Siemienski, K., Muhlenbeck, F., Durkop, H., Pfizenmaier, K., Scheurich, P., Wajant, H., 1998. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J. Immunol.* 161, 3136.
- Xu, H., Sethi, J.K., Hotamisligil, G.S., 1999. Transmembrane tumor necrosis factor (TNF)-alpha inhibits adipocyte differentiation by selectively activating TNF receptor 1. *J. Biol. Chem.* 274, 26287.
- Yamamoto, Y., Tsutsumi, Y., Yoshioka, Y., Nishibata, T., Kobayashi, K., Okamoto, T., Mukai, Y., Shimizu, T., Nakagawa, S., Nagata, S., Mayumi, T., 2003. Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nat. Biotechnol.* 21, 546.

## RESEARCH PAPER

# Comparative study on transduction and toxicity of protein transduction domains

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**Background and purpose:** Protein transduction domains (PTDs), such as Tat, antennapedia homeoprotein (Antp), Rev and VP22, have been extensively utilized for intracellular delivery of biologically active macromolecules *in vitro* and *in vivo*. There is little known, however, about the relative transduction efficacy, cytotoxicity and internalization mechanism of individual PTDs. **Experimental approach:** We examined the cargo delivery efficacies of four major PTDs (Tat, Antp, Rev and VP22) and evaluated their toxicities and cell internalizing pathways in various cell lines.

**Key results:** The relative order of the transduction efficacy of these PTDs conjugated to fluorescein was Rev > Antp > Tat > VP22, independent of cell type (HeLa, HaCaT, A431, Jurkat, MOLT-4 and HL60 cells). Antp produced significant toxicity in HeLa and Jurkat cells, and Rev produced significant toxicity in Jurkat cells. Flow cytometric analysis demonstrated that the uptake of PTD-fluorescein conjugate was dose-dependently inhibited by methyl- $\beta$ -cyclodextrin, cytochalasin D and amiloride, indicating that all four PTDs were internalized by the macropinocytotic pathway. Accordingly, in cells co-treated with 'Tat-fused' endosome-disruptive HA2 peptides (HA2-Tat) and independent PTD-fluorescent protein conjugates, fluorescence spread throughout the cytosol, indicating that all four PTDs were internalized into the same vesicles as Tat.

**Conclusions and implications:** These findings suggest that macropinocytosis-dependent internalization is a crucial step in PTD-mediated molecular transduction. From the viewpoint of developing effective and safe protein transduction technology, although Tat was the most versatile carrier among the peptides studied, PTDs should be selected based on their individual characteristics.

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**Keywords:** protein transduction domains; Tat; antennapedia; Rev; VP22; macropinocytosis

**Abbreviations:** PTD, protein transduction domain; Antp, antennapedia

## Introduction

A strong focus of research in the post-genomic era is the development of effective therapies for refractory diseases such as cancer and neurodegenerative syndromes (Rhodes and Chinnaiyan, 2005; Brusic *et al.*, 2007; Drabik *et al.*, 2007). Because the therapeutic targets of these diseases generally exist inside the cell, it is necessary to establish drug delivery methods that transfer macromolecules, such as therapeutic proteins or peptide-based drugs, across the cellular membrane (Nori and Kopecek, 2005; Murriel and Dowdy, 2006; Borsello and Forloni, 2007).

Protein transduction is a recently developed method for delivering biologically active proteins directly into mammalian cells with high efficiency (Hawiger, 1999; Schwarze *et al.*, 2000). Recombinant technology is used to modify the biophysical properties of proteins and peptides, particularly with respect to their cell permeability, using the so-called protein transduction domains (PTDs) (Nagahara *et al.*, 1998; Rojas *et al.*, 1998; Schwarze *et al.*, 1999). The HIV-1-derived Tat peptide renders various macromolecules cell permeable. Although the initial reports suggested that protein transduction is energy and temperature independent, these characteristics are now mostly attributed to phenomena such as fixation artefacts (Richard *et al.*, 2003). More recent data indicate that basic PTDs such as Tat enter the cells through a macropinocytotic pathway that is universally active in all cells (Wadia *et al.*, 2004; Kaplan *et al.*, 2005). A series of events involves Tat attachment to an anionic cell surface,

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followed by the association of these complexes with lipid rafts, which triggers dynamin-independent macropinocytosis. After internalization, the endosome pH falls and Tat apparently destabilizes the membranes, which results in a small amount of the endosome contents escaping into the cytosol. The released fraction of Tat can then exert its biologic activity. Consistent with this model, Tat delivery is enhanced by the influenza virus haemagglutinin fusogenic motif, which further destabilizes endosomal membranes at a low pH (Han *et al.*, 2001; Skehel *et al.*, 2001). In cells cotreated with Tat-conjugated HA2 (HA2-Tat), a greater proportion of endocytosed Tat-fused Cre recombinase escapes into the cytoplasm (Wadia *et al.*, 2004). Other studies suggest that certain cell types might incorporate Tat constructs by clathrin- or caveolin-dependent endocytosis, raising the possibility that transport varies according to the cargo or cell type (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003; Richard *et al.*, 2005). In addition to the basic Tat peptide, there are other proteins (fragments), such as antennapedia (Antp), Rev and VP22, that enhance cellular uptake of proteins (Table 1) (Derossi *et al.*, 1994; Elliott and O'Hare, 1997; Futaki *et al.*, 2001; Joliot and Prochiantz, 2004). These four well-known PTDs facilitate the delivery of various biomacromolecules into the cell, but few studies have examined their relative efficacy.

In the present study, we evaluated the potency and internalizing pathway of four major PTDs to optimize protein transduction technology and to clarify the mechanisms of action. We also evaluated the cytotoxicity of these four PTDs as this is crucial to their utility as effective biomacromolecule carriers. These analyses may help not only to elucidate the mechanism by which the four peptides facilitate the cellular uptake of biomacromolecules, but also provide criteria for their proper use.

## Materials and methods

### Cell lines

HeLa cells (human cervical carcinoma cells) and A431 cells (human epithelial carcinoma cells) were obtained from the American Type Culture Collection (Manassas, VA, USA). HaCaT cells (human keratinocyte cells) were kindly provided by Dr S Inui, Osaka University. Jurkat cells (human leukaemia cells) and MOLT-4 cells (human leukaemia cells) were kindly provided by Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). HL60 cells (human promyelocytic leukaemia cells) were obtained from the Japanese Collection of Research Bioresources (JCRB; Osaka, Japan). HeLa cells were cultured in minimal essential medium (MEM $\alpha$ ; Wako Pure Chemicals, Osaka, Japan) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. A431 cells and HaCaT cells were maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemicals) supplemented with 10% FBS, 1% L-glutamine and antibiotics. Jurkat cells and MOLT-4 cells were maintained in RPMI-1640 medium (Wako Pure Chemicals) supplemented with 10% FBS and antibiotics. HL60 cells were maintained in RPMI-1640 medium (Wako Pure Chemicals) supplemented with

**Table 1** Protein sequences of the PTDs evaluated

PTD	Origin	Sequence	pI
Tat	HIV-1	GRKKRRQRRRPPQ	12.70
Antp	<i>Drosophila</i>	RQIKIWFAQNRRMKWKKK	12.31
Rev	HIV-1	TRQARRNRRRRWRERQF	12.60
VP22	HSV	NAKTRRHERRRKLAIER	12.01

The basic amino acids in each sequence are shown in bold.

20% FBS and antibiotics. All cells were cultured at 37 °C in 5% CO<sub>2</sub>.

### Synthetic peptides

All peptides used in this study were purchased from the Toray Research Center Inc. (Tokyo, Japan) and had purities above 90%, which was confirmed by high-performance liquid chromatography analysis and mass spectroscopy. The sequences of these peptides were GRKKRRQRRRPPQK-FAM (FAM = carboxyfluorescein) for Tat-conjugated FAM (Tat-FAM), RQIKIWFAQNRRMKWKKK-FAM for Antp-conjugated FAM (Antp-FAM), TRQARRNRRRRWRERQK-FAM for Rev-conjugated FAM (Rev-FAM), NAKTRRHERRRKLAIERK-FAM for VP22-conjugated FAM (VP22-FAM), YGRKKRRQRRRK-biotin for Tat-conjugated biotin, RQIKIWFAQNRRMKWKKK-biotin for Antp-conjugated biotin, TRQARRNRRRRWRERQK-biotin for Rev-conjugated biotin, NAKTRRHERRRKLAIERK-biotin for VP22-conjugated biotin and GLFEAIEGFIENGWEGMIDGWYGYGRKKRRQRRR for HA2-conjugated Tat (HA2-Tat). The individual PTD sequences are underlined.

### Flow cytometric analysis

HeLa cells, HaCaT cells and A431 cells were cultured in 24-well plates (Nalge Nunc International, Naperville, IL, USA) at  $5.0 \times 10^4$  cells per well in culture medium and incubated for 24 h at 37 °C. Jurkat cells, MOLT-4 cells and HL60 cells were cultured in 24-well plates (Nalge Nunc International) at  $1.0 \times 10^5$  cells per well in Opti-MEM I (Invitrogen, Carlsbad, CA, USA). After aspirating the media, FAM-conjugated PTD (PTD-FAM) (10  $\mu$ M) was added to the cells in Opti-MEM I and the culture dishes were incubated for an additional 3 h. Following incubation, the cells were washed with phosphate-buffered saline and incubated for 5 min with 0.1% trypsin to detach them and to remove surface-bound peptides. After incubation, 2 vol of 10% FBS-containing culture medium was added to stop the trypsin activity and to detach the cells completely. The cell suspension was centrifuged at 800 g, washed with phosphate-buffered saline, centrifuged again and resuspended in 500  $\mu$ l of 0.4% paraformaldehyde. Fluorescence was analysed on a FACSCalibur flow cytometer, and data were analysed using CellQuest software (both from Becton Dickinson, San Jose, CA, USA). In the low-temperature uptake experiment, cells were preincubated at 4 °C for 1 h in Opti-MEM I prior to adding the PTDs, and all buffers and solutions were equilibrated to 4 °C. To analyse the internalization mechanism, HeLa cells were pretreated for 30 min in Opti-MEM I medium with 0–5 mM methyl- $\beta$ -cyclodextrin, 0–2.5  $\mu$ M

cytochalasin D or 0–5 mM amiloride (all from Sigma-Aldrich, St Louis, MO, USA). After the PTD-FAMs were added, cells were maintained for 1 h (30 min for amiloride) in the presence of inhibitors and washed several times with phosphate-buffered saline. As a control, the cellular uptake of transferrin fluorescein isothiocyanate (Invitrogen) was also monitored.

#### Cell proliferation assay

Cell viability was determined using a WST-8 assay kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. The assay is based on the cleavage of the tetrazolium salt WST-8 to formazan by cellular mitochondrial dehydrogenase. HeLa cells were cultured in 96-well plates (Nalge Nunc International) at  $5.0 \times 10^3$  cells per well in MEM $\alpha$  and incubated for 24 h at 37 °C. Jurkat cells were cultured in 96-well plates at  $1.0 \times 10^4$  cells per well in Dulbecco's modified Eagle's medium. The cells were treated with various concentrations of PTD-biotin. After 24 h incubation, cell viability was measured using the WST-8 assay kit.

#### Membrane integrity assay

The lactate dehydrogenase (LDH) leakage assay was used to quantify the membrane integrity of the PTD-treated cells. This assay detects the amount of LDH released into the culture media as a result of plasma membrane disruption after PTD treatment. HeLa cells were cultured in 96-well plates (Nalge Nunc International) at  $5.0 \times 10^3$  cells per well in MEM $\alpha$  and incubated for 24 h at 37 °C. Jurkat cells were cultured in 96-well plates at  $1.0 \times 10^4$  cells per well in Dulbecco's modified Eagle's medium. Each cell type was treated with various concentrations of PTD-biotin. After 3 h incubation, the LDH release activity of the peptides was measured using an LDH cytotoxicity test (Wako) according to the manufacturer's instructions.

#### Expression and purification of PTD-fused Venus protein

The Venus (variant of yellow fluorescent protein) DNA sequence was kindly provided by Dr A Miyawaki (RIKEN Brain Science Institute). The Tat-Venus DNA sequence was amplified by PCR. At the 5' end, the primer sequence 5'-TTTAAGAAGGAGATATACATATGGCTTACGGTCGTAAAAA CGTCGCCAGCGTCGCCGTGGTGGCGGCGGTTCCCTCGA GCACCACCATCACCACCATGTGAGCAAGGGCGAGGAGC TGTTTAC-3' introduced an *Nde* I site and Tat sequence and at the 3' end, the primer sequence 5'-GCTTTGTTAGCAG CCGAATTCTTACTGTACAGCTCGTCCATGCCGAGAGTGA TC-3' introduced an *Eco* RI site. The PCR product was digested with *Nde* I and *Eco* RI and inserted into a protein expression plasmid. Other plasmids expressing Antp-, Rev- or VP22-Venus recombinant proteins were constructed by replacing the Tat-coding region in the Tat-Venus plasmid with the Antp, Rev or VP22 sequences using the *Nde* I and *Xho* I restriction sites. These sequences were obtained by annealing the following oligonucleotides with protruding single-strand DNA corresponding to the *Nde* I and *Xho* I sites:

Antp sense, 5'-TATGGCTCGTCAGATCAAAATCTGGTTCCA GAATCGTCGTATGAAGTGGAAAAAGGTGGCGGCGGTTCC-3'; Antp antisense, 5'-TCGAGGGAACCGCCGCCACCTT TTTCCACTTCATACGACGATTCTGGAACAGATTTTGATC TGACGAGCCA-3'; Rev sense, 5'-TATGGCTACCCGTCAGGC TCGTCGTAATCGTCGTCGTTGGCGTGAACGTCAGCGT GGTGGCGGCGGTTCCC-3'; Rev antisense, 5'-TCGAGGGAA CCGCCGCCACCACGCTGACGTTACGCCAACGACGACGA CGATTACGACGAGCCTGACGGGTAGCCA-3'; VP22 sense, 5'-TATGGCTAACGCTAAAACCCGTCGTCACGAACGTCGTCG TAAACTGGCTATCGAACGTGGTGGCGGCGGTTCCC-3'; VP22 antisense, 5'-TCGAGGGAACCGCCGCCACCACGTTGATAG CCAGTTTACGACGACGTTTCGTGACGACGGGTTTAGCGT TAGCCA-3'. The plasmids, except for the Antp-Venus expression vector, were transformed into *Escherichia coli* BL21 Star (DE3) (Invitrogen). The Antp-Venus-expressing vector was transformed into BL21 Star (DE3), in which the plasmid-expressing chaperone (pGro7) was pretransformed. Transformed *E. coli* was cultured and the cell paste was suspended in BugBuster Master Mix (Novagen, Darmstadt, Germany) and centrifuged. PTD-Venus was recovered in the supernatant and purified by His-tag affinity purification and gel filtration chromatography.

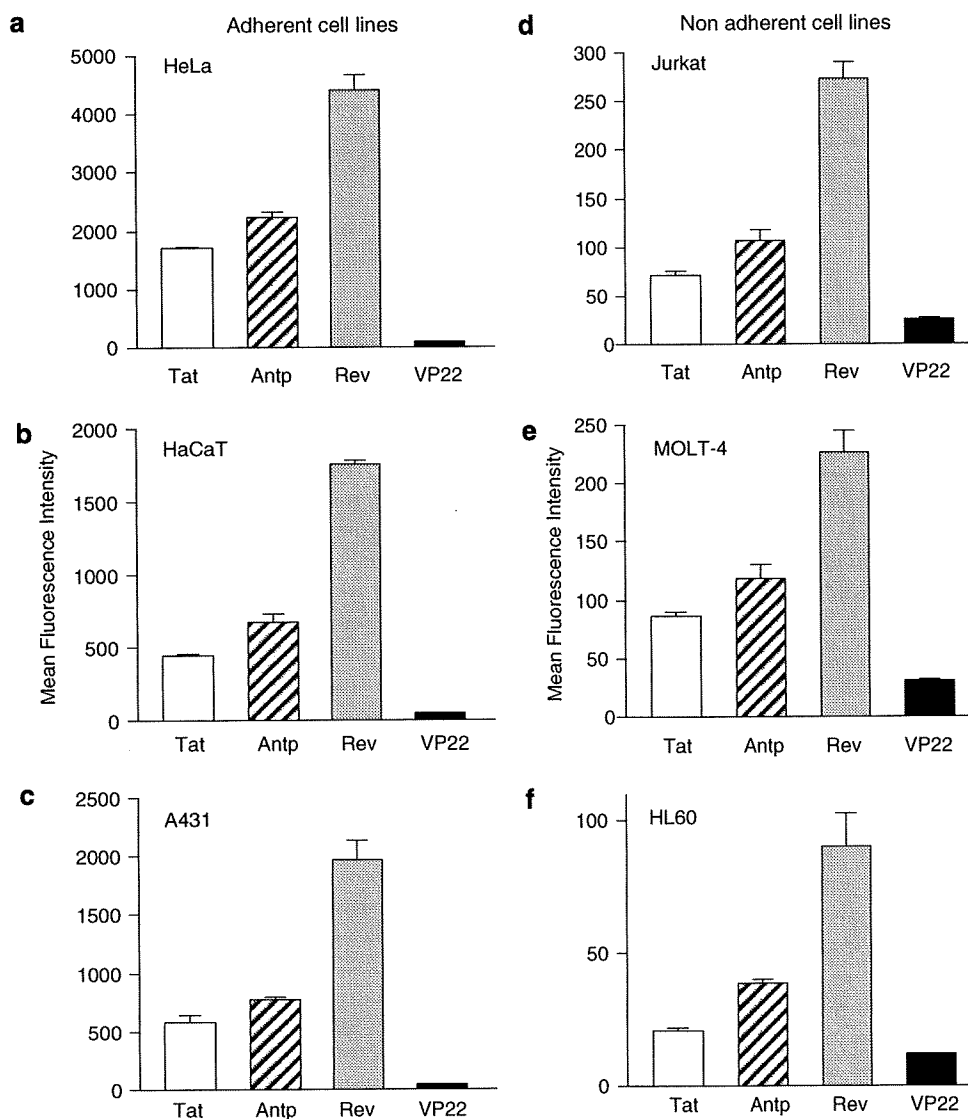
#### Confocal laser scanning microscopy analysis

HeLa cells were cultured on Lab-Tek II Chambered Cover-glass (Nalge Nunc International) at  $3.0 \times 10^4$  cells per well in MEM $\alpha$  supplemented with 10% FBS and incubated for 24 h at 37 °C. Internalization of PTD-FAM or PTD-Venus was performed as follows: HeLa cells were treated with PTD-FAM or PTD-Venus (10  $\mu$ M) in Opti-MEM I containing 100 ng ml<sup>-1</sup> Hoechst 33342 (Invitrogen) and 6  $\mu$ g ml<sup>-1</sup> FM4-64 (Invitrogen). After incubation at 37 °C for 3 h, the medium was exchanged with fresh medium and fluorescence was observed by confocal laser scanning microscopy (Leica Microsystems GmbH, Wetzlar, Germany) without cell fixation. For cotreatment with HA2-Tat, HeLa cells were cotreated with PTD-FAM (10  $\mu$ M) and HA2-Tat (2  $\mu$ M) in Opti MEM I containing 100 ng ml<sup>-1</sup> Hoechst 33342. After incubation at 37 °C for 3 h, the medium was exchanged with fresh medium and fluorescence was observed by confocal laser scanning microscopy without cell fixation.

## Results

#### Comparison of transduction efficiency and cytotoxicity of four PTDs

To confirm the intracellular translocation activity of the four selected PTDs, we evaluated the transduction efficiency of Tat-, Antp-, Rev- and VP22-FAM in six cell lines (adherent: HeLa, HaCaT and A431 cells; nonadherent: Jurkat, MOLT-4 and HL60 cells) using flow cytometric analysis (Figure 1). These PTDs contain a large number of basic amino acids (Table 1) and their cationic properties are thought to be important for cell membrane penetration (Futaki *et al.*, 2001; Chauhan *et al.*, 2007). Their positive charge, however, causes them to adsorb nonspecifically to negatively charged cell

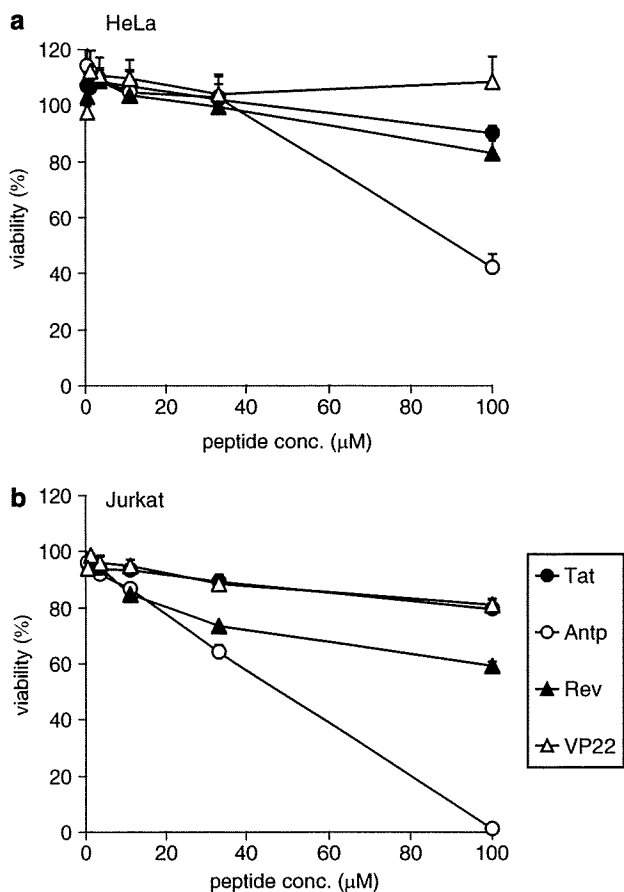


**Figure 1** Comparison of the cellular uptake of protein transduction domains (PTDs). FAM-labelled Tat (white column), antennapedia (Antp; hatched column), Rev (grey column) and VP22 (black column) were incubated with six cell lines: HeLa (a), HaCaT (b), A431 (c), Jurkat (d), MOLT-4 (e) and HL60 (f) at  $10\ \mu\text{M}$  for 3 h. After trypsin treatment to digest PTDs adsorbed on the cell surface, the PTD-transduced cells were harvested and analysed by flow cytometry. Note that the y axis scales for the adherent cell lines are markedly different from that for the nonadherent cell lines. Data shown are the mean  $\pm$  s.d. of triplicate assays.

membranes (Richard *et al.*, 2003). For this reason, the cells were treated with excess trypsin to eliminate nonspecific plasma membrane binding of the PTDs prior to measurement.

The relative order of their translocation efficiency (Rev > Antp > Tat > VP22), which was based on mean fluorescence, was independent of the cell type (that is, adherent or nonadherent). Furthermore, using PTD-fused Venus, we confirmed that Rev had the highest transduction efficiency (data not shown). Equally important, the overall translocation efficiency of the PTDs depended markedly on whether the cells were adherent (HeLa, HaCaT and A431 cells) or nonadherent (Jurkat, MOLT-4 and HL60 cells). The transduction efficiency was much higher in the adherent cell lines compared with the nonadherent cell lines (Figure 1); note that that the fluorescence (uptake) was about 8- to 25-fold greater in the adherent, than in nonadherent, cell lines.

The cytotoxic properties of the four PTDs were evaluated in adherent (HeLa) and nonadherent (Jurkat) cells. To assess the long-term changes in proliferation, mitochondrial dehydrogenase activity was measured using a WST-8 assay 24 h after PTD treatment. In HeLa cells, there was a remarkable decrease in cell viability when the cells were incubated with Antp at  $100\ \mu\text{M}$ , whereas other PTDs were not cytotoxic at the higher concentrations (Figure 2a). In contrast in Jurkat cells, Antp was extremely cytotoxic in a dose-dependent manner and Rev reduced cell proliferation by approximately 40% (Figure 2b). Previous reports indicated that amphipathic peptides, such as transportan, induced cytotoxicity by perturbing the cellular membrane (Hallbrink *et al.*, 2001; Jones *et al.*, 2005; Saar *et al.*, 2005; El-Andaloussi *et al.*, 2007). Thus, the membrane integrity of PTD-treated cells was also measured using an LDH leakage assay. Antp

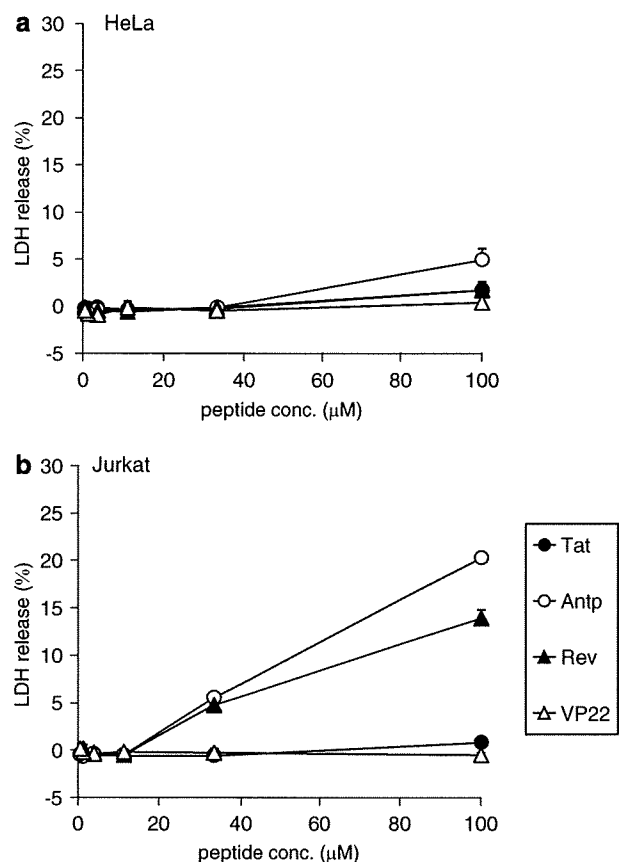


**Figure 2** Viability of protein transduction domain (PTD)-treated cells. HeLa cells (a) and Jurkat cells (b) were incubated with serially diluted biotin-conjugated Tat, antennapedia (Antp), Rev and VP22 at 37 °C. After 24 h, cell viability was analysed using a WST-8 assay. Data shown are the mean ± s.d. of triplicate assays.

and Rev induced significant LDH leakage in Jurkat cells, but only low LDH leakage was detected in Antp-treated HeLa cells (Figure 3). The membrane-perturbing effect of Antp and Rev contributed to the uptake of peptides, which are shown in Figure 1. Jurkat cells appear more sensitive to Antp or Rev treatment than HeLa cells; this difference in cytotoxicity and translocation efficiency may indicate a difference in the PTD-uptake mode.

*Intracellular transduction mechanism of PTDs*

The results of *in vitro* studies suggest that PTDs enter the cell via an energy-dependent endocytotic pathway (Lundberg *et al.*, 2003; Richard *et al.*, 2003). In particular, studies using various macropinocytosis inhibitors, such as methyl-β-cyclodextrin, to deplete cholesterol from the membrane (Grimmer *et al.*, 2002; Liu *et al.*, 2002), cytochalasin D, to inhibit F-actin elongation (Sampath and Pollard, 1991), or amiloride, to inhibit the Na<sup>+</sup>-H<sup>+</sup> exchanger (West *et al.*, 1989), indicate that Tat is taken up into the cell via lipid raft-dependent macropinocytosis. To the best of our knowledge, however, few comparative studies have analysed the cellular uptake pathway of the four PTDs discussed in this paper. Therefore, we used flow cytometry analysis to determine

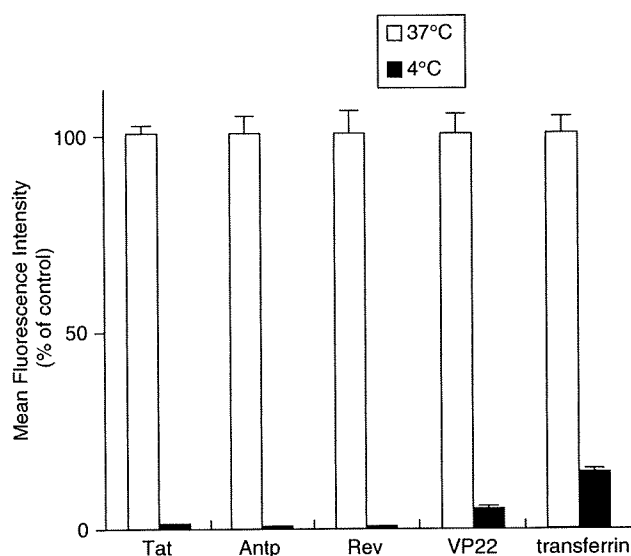


**Figure 3** Membrane integrity of protein transduction domain (PTD)-treated cells. HeLa cells (a) and Jurkat cells (b) were incubated with serially diluted biotin-conjugated Tat, antennapedia (Antp), Rev and VP22 at 37 °C. After 3 h, the release of lactate dehydrogenase (LDH) was analysed. Data shown are the mean ± s.d. of triplicate assays.

whether PTD uptake is energy dependent or occurs via lipid raft-mediated macropinocytosis. First, we treated cells with PTD-FAM at 37 or 4 °C and then measured cell fluorescence (Figure 4). At 4 °C, transferrin, which enters cells by clathrin-dependent endocytosis (Schmid, 1997), inhibited the transduction efficiency compared with that at 37 °C. All four PTDs had low transduction ability at 4 °C, indicating that their cellular uptake was energy dependent. We next examined the PTD-FAM uptake efficiency in methyl-β-cyclodextrin-, cytochalasin D- and amiloride-treated HeLa cells. These cell treatments inhibited PTD-FAM incorporation in a dose-dependent manner, but transferrin was not affected (Figure 5). Furthermore, in HeLa cells treated with PTD-FAM, only punctuate fluorescence was observed using confocal laser scanning microscopic analysis (Figure 6). These results indicated that all the PTDs evaluated in this study enter the cell through the macropinocytotic pathway and that most of them were trapped in intracellular vesicles, the macropinosomes.

*Intracellular localization of PTD-protein conjugates*

We next examined the intracellular behaviour of the individual PTDs in more detail. To investigate whether

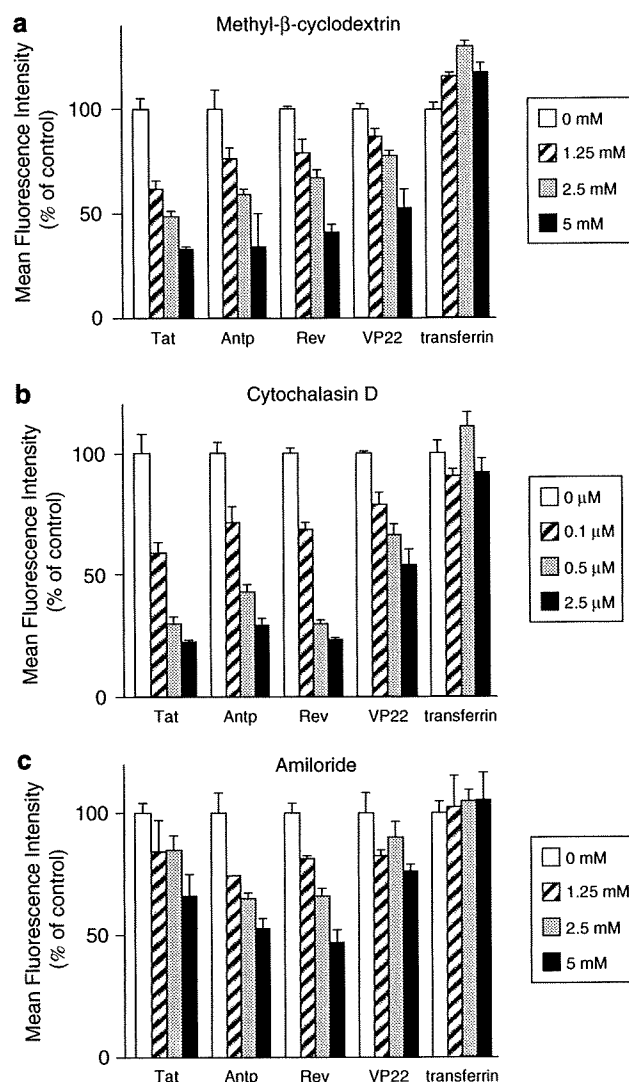


**Figure 4** Effects of temperature on protein transduction domain (PTD) transduction efficiency. HeLa cells were preincubated at 37 or 4°C for 1 h prior to adding FAM-labelled PTDs or fluorescein isothiocyanate-labelled transferrin for 3 h. Cells were washed in trypsin and analysed by flow cytometry. Data shown are the mean  $\pm$  s.d. of triplicate assays.

individual PTDs are located in the same vesicles, we used Tat-fused HA2 peptide (HA2-Tat), an influenza virus-derived endosome-disrupting peptide. HA2-Tat improves the activity of Tat-fused Cre recombinase (Wadia *et al.*, 2004). Because HA2 alone cannot enter the cell, HA2-Tat is thought to enter the cell in a Tat-dependent manner and to disrupt the membrane of endosomal vesicles in which the Tat cargo is trapped. Thus, if Antp, Rev and VP22 are trapped in the same vesicles as Tat, the fluorescence should spread throughout the cytosol following cotreatment of the cells with HA2-Tat. As predicted, in HeLa cells cotreated with Antp-, Rev- or VP22-Venus and HA2-Tat, the Venus-derived fluorescence spread throughout the cytosol, whereas in the cells treated with Antp-, Rev- or VP22-Venus alone, only punctuate fluorescence was observed (Figure 7). These results suggested that all the PTDs evaluated in this study entered the cell through a macropinocytotic pathway and were trapped in the same vesicles as Tat.

## Discussion

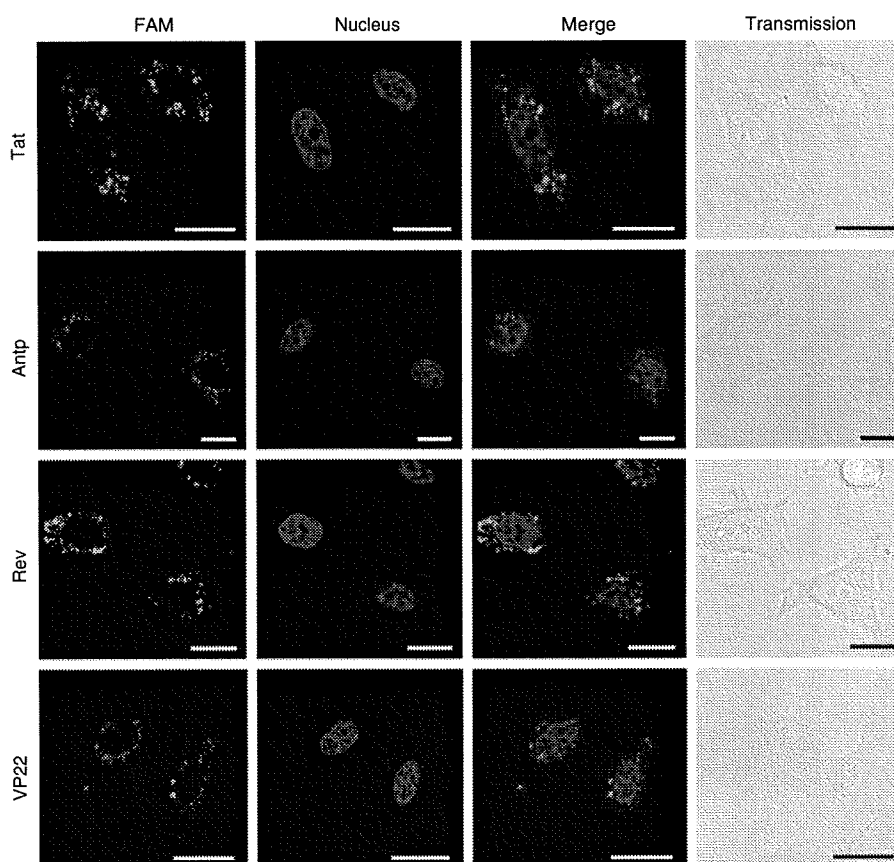
In the present study, we have systematically compared PTD-mediated molecular transduction mechanisms. Our findings indicated that individual PTDs have different levels of transduction efficiency and cytotoxicity, suggesting that PTDs are internalized into live cells via different mechanisms. We also examined the internalization pathway and intracellular localization of Tat, Antp, Rev and VP22. Unexpectedly, all the PTDs evaluated in this study entered the cell through the macropinocytotic pathway and were trapped in the same vesicles as Tat. The finding that the intracellular transduction pathways of the four PTDs were the same suggests that the method of cell internalization does not contribute to the



**Figure 5** Effects of endocytosis inhibitors on transduction efficiency of protein transduction domains (PTDs). HeLa cells were pretreated with a range of concentrations of (a) methyl-β-cyclodextrin, (b) cytochalasin D or (c) amiloride for 30 min prior to adding FAM-labelled PTDs or fluorescein isothiocyanate-labelled transferrin for 1 h (a and b) or 30 min (c). Cells were washed in trypsin and analysed by flow cytometry. Data shown are the mean  $\pm$  s.d. of triplicate assays.

differences in the PTD transduction efficiency or cytotoxicity. Although the reason for this phenomenon is not clear, we speculate that the primary structure of the individual PTDs or the cell surface proteins that interact with the individual PTDs contribute to the differences in their transduction efficiency and cytotoxicity.

The initial step in the mechanism of cellular entry of PTDs is thought to be the strong ionic interaction between the amino-acid residues of the PTDs and the plasma membrane constituents. Because the translocation is solely physically mediated, the charge distribution and amphipathicity of the peptide and its interaction with the plasma membrane is critical (Pujals *et al.*, 2006). Although most PTDs, if not all, contain a large number of basic amino acids, such as arginine or lysine, the theoretical isoelectric point (pI) value of each PTD used in this study was essentially identical (Tat, Antp,



**Figure 6** Intracellular behaviour of protein transduction domain (PTD)-FAM in living cells. HeLa cells were treated with 10  $\mu\text{M}$  PTD-FAM for 3 h. Fluorescence images were acquired using confocal laser scanning microscopy and the signals were merged electronically. The nucleus was counterstained with Hoechst 33342 (blue). From top to bottom: Tat-, antennapedia (Antp)-, Rev- and VP22-FAM. From left to right: FAM (green), nucleus (blue), merged fluorescence and transmission image. Scale bars in each microphotograph indicate 20  $\mu\text{m}$ .

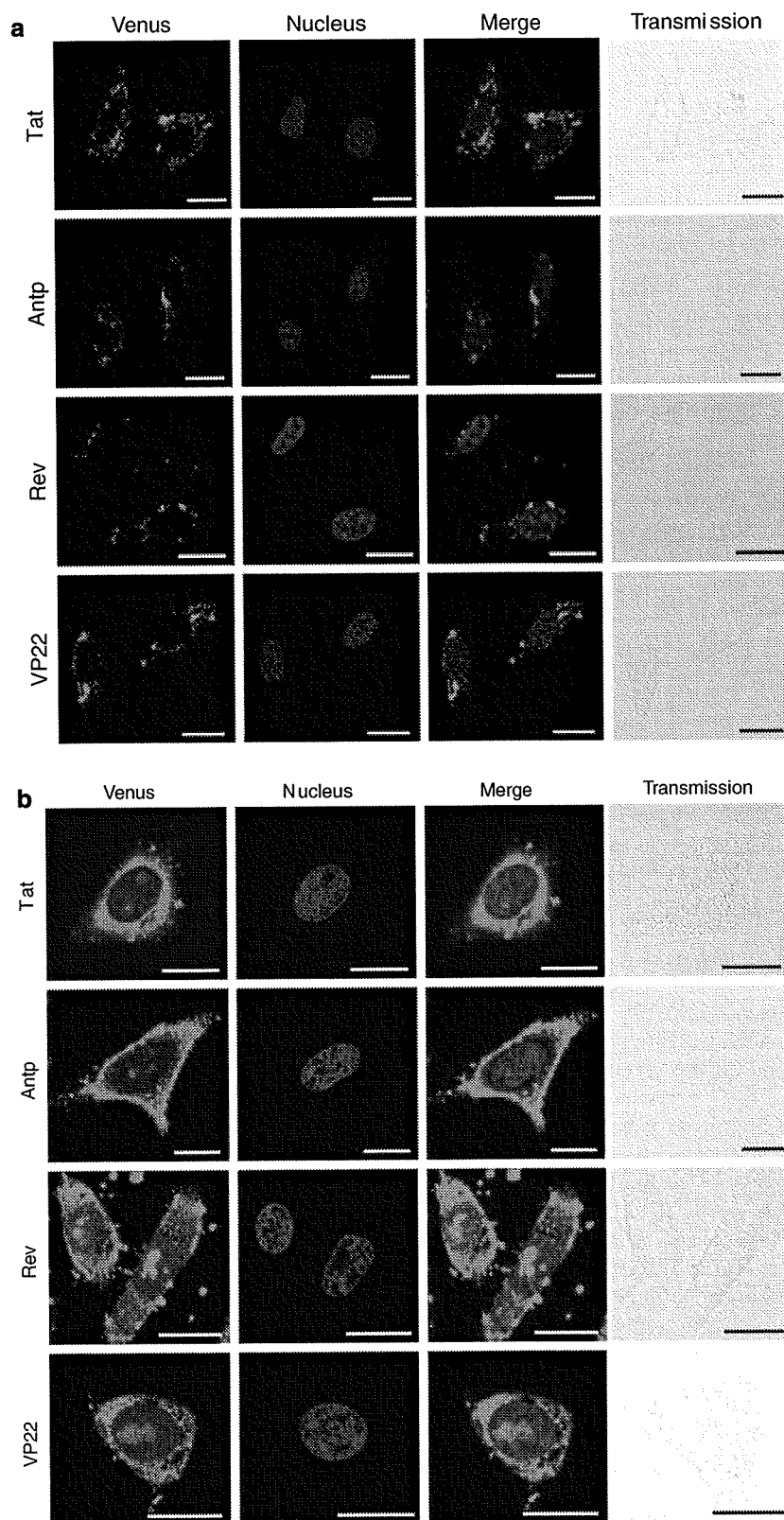
Rev and VP22 have pI values of 12.70, 12.31, 12.60 and 12.01, respectively). Therefore, the internalization efficiency does not appear to depend on the cationic features of the PTDs.

The amphipathicity of the carrier is probably responsible not only for the strong interaction with the lipid membranes (Yandek *et al.*, 2007), but also for the disruption of the cellular membrane, which results in cell death (Hallbrink *et al.*, 2001; Jones *et al.*, 2005; Saar *et al.*, 2005; El-Andaloussi *et al.*, 2007). In terms of cytotoxicity, our data indicate that Antp and Rev both disrupt the membrane (Figure 3), but Rev does not contain an amphipathic structure. Furthermore, there was no correlation between hydrophobicity and transduction efficiency. Thus, differences in the PTD-mediated transduction efficiency and cytotoxicity might be due to the molecular weight or pI of the conjugated cargo.

The cellular events required for internalization, however, differ between reports and are often conflicting. The first mechanistic studies led to the proposal that PTD internalization occurs rapidly in a receptor- and energy-independent manner, perhaps by destabilizing the lipid bilayer or by the formation of inverted micelles with subsequent release of their contents within the intracellular space (Berlose *et al.*, 1996). More recently, an active mechanism based on vesicular uptake was proposed as the general mode of cell

internalization of PTDs. In our experiment, although all four PTDs tended to be present in the same vesicles, the detailed mechanism for this colocalization is not yet known. It has been suggested that PTD internalization requires cell surface heparan sulphate proteoglycans (Tyagi *et al.*, 2001; Console *et al.*, 2003; Ziegler and Seelig, 2004). Because Tat interacts electrostatically with heparan sulphate proteoglycan present on the cell surface, it is possible that some PTDs are taken into the same vesicles when they interact with one heparan sulphate proteoglycan. In contrast, as shown in Figure 7, although fluorescence was observed throughout the cytosol, punctate fluorescence was also observed when the cells were cotreated with PTD-Venus and HA2-Tat. This finding suggested that the PTDs did not all exist in the same vesicles and that some PTDs entered the cell through another pathway. This is just speculation, however, and we are now using proteome analysis, such as liquid chromatography coupled with mass spectrometry or two-dimensional gel electrophoresis, to examine whether there are individual cell surface receptors for different PTDs.

In summary, our data suggest that Antp, Rev, VP22 and Tat cross the plasma membrane and reach the macropinosomes via different mechanisms. Our findings also indicate that several issues, such as endosome entrapment and low cell specificity, which limit the therapeutic activity of the cargo,



**Figure 7** Intracellular behaviour of protein transduction domain (PTD)-Venus in living cells. HeLa cells were treated with 10  $\mu$ M PTD-Venus alone (**a**) or 10  $\mu$ M HA2-Tat (**b**) for 3 h. Fluorescence images were acquired using confocal laser scanning microscopy and the signals were merged electronically. The nucleus was counterstained with Hoechst 33342 (blue). From top to bottom: Tat-, antennapedia (Antp)-, Rev- and VP22-Venus. From left to right: Venus (green), nucleus (blue), merged fluorescence and transmission image. Scale bars in each microphotograph indicate 20  $\mu$ m.

must be overcome before effective PTD-based drug delivery carriers can be fully developed. We previously reported that cotreatment with HA2-Tat enhances the cytosolic release of Tat-fused peptide-blockers and their biological activities, thereby overcoming the issue of endosome entrapment (Sugita *et al.*, 2007). Furthermore, although the transduction mechanism of PTDs is not yet well understood, these differences led us to explore the possibility of creating novel PTDs. We successfully created novel PTDs that have higher transduction efficiencies than Tat, using a unique phage display-based screening strategy that we previously developed (Mukai *et al.*, 2006; Kamada *et al.*, 2007). Moreover, based on our PTD-screening system, we are currently working to create more useful PTDs with cell type specificity.

### Conflict of interest

The authors state no conflict of interest.

### References

- Berlose JP, Convert O, Derossi D, Brunissen A, Chassaing G (1996). Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments. *Eur J Biochem* 242: 372–386.
- Borsello T, Forloni G (2007). JNK signalling: a possible target to prevent neurodegeneration. *Curr Pharm Des* 13: 1875–1886.
- Brusic V, Marina O, Wu CJ, Reinherz EL (2007). Proteome informatics for cancer research: from molecules to clinic. *Proteomics* 7: 976–991.
- Chauhan A, Tikoo A, Kapur AK, Singh M (2007). The taming of the cell penetrating domain of the HIV Tat: myths and realities. *J Control Release* 117: 148–162.
- Console S, Marty C, Garcia-Echeverria C, Schwendener R, Ballmer-Hofer K (2003). Antennapedia and HIV transactivator of transcription (TAT) 'protein transduction domains' promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J Biol Chem* 278: 35109–35114.
- Derossi D, Joliot AH, Chassaing G, Prochiantz A (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* 269: 10444–10450.
- Drabik A, Bierzynska-Krzysik A, Bodzon-Kulakowska A, Suder P, Kotlinska J, Silberring J (2007). Proteomics in neurosciences. *Mass Spectrom Rev* 26: 432–450.
- El-Andaloussi S, Jarver P, Johansson HJ, Langel U (2007). Cargo dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study. *Biochem J* 407: 285–292.
- Elliott G, O'Hare P (1997). Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* 88: 223–233.
- Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F (2003). Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Mol Ther* 8: 284–294.
- Fittipaldi A, Ferrari A, Zoppe M, Arcangeli C, Pellegrini V, Beltram F *et al.* (2003). Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J Biol Chem* 278: 34141–34149.
- Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K *et al.* (2001). Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* 276: 5836–5840.
- Grimmer S, van Deurs B, Sandvig K (2002). Membrane ruffling and macropinocytosis in A431 cells require cholesterol. *J Cell Sci* 115: 2953–2962.
- Hallbrink M, Floren A, Elmquist A, Pooga M, Bartfai T, Langel U (2001). Cargo delivery kinetics of cell-penetrating peptides. *Biochim Biophys Acta* 1515: 101–109.
- Han X, Bushweller JH, Cafiso DS, Tamm LK (2001). Membrane structure and fusion-triggering conformational change of the fusion domain from influenza hemagglutinin. *Nat Struct Biol* 8: 715–720.
- Hawiger J (1999). Noninvasive intracellular delivery of functional peptides and proteins. *Curr Opin Chem Biol* 3: 89–94.
- Joliot A, Prochiantz A (2004). Transduction peptides: from technology to physiology. *Nat Cell Biol* 6: 189–196.
- Jones SW, Christison R, Bundell K, Joyce CJ, Brockbank SM, Newham P *et al.* (2005). Characterisation of cell-penetrating peptide-mediated peptide delivery. *Br J Pharmacol* 145: 1093–1102.
- Kamada H, Okamoto T, Kawamura M, Shibata H, Abe Y, Ohkawa A *et al.* (2007). Creation of novel cell-penetrating peptides for intracellular drug delivery using systematic phage display technology originated from Tat transduction domain. *Biol Pharm Bull* 30: 218–223.
- Kaplan IM, Wadia JS, Dowdy SF (2005). Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release* 102: 247–253.
- Liu NQ, Lossinsky AS, Popik W, Li X, Gujuluva C, Kriederman B *et al.* (2002). Human immunodeficiency virus type 1 enters brain microvascular endothelia by macropinocytosis dependent on lipid rafts and the mitogen-activated protein kinase signaling pathway. *J Virol* 76: 6689–6700.
- Lundberg M, Wikstrom S, Johansson M (2003). Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther* 8: 143–150.
- Mukai Y, Sugita T, Yamato T, Yamanada N, Shibata H, Imai S *et al.* (2006). Creation of novel protein transduction domain (PTD) mutants by a phage display-based high-throughput screening system. *Biol Pharm Bull* 29: 1570–1574.
- Murriel CL, Dowdy SF (2006). Influence of protein transduction domains on intracellular delivery of macromolecules. *Expert Opin Drug Deliv* 3: 739–746.
- Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA *et al.* (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat Med* 4: 1449–1452.
- Nori A, Kopecek J (2005). Intracellular targeting of polymer-bound drugs for cancer chemotherapy. *Adv Drug Deliv Rev* 57: 609–636.
- Pujals S, Fernandez-Carneado J, Lopez-Iglesias C, Kogan MJ, Giralt E (2006). Mechanistic aspects of CPP-mediated intracellular drug delivery: relevance of CPP self-assembly. *Biochim Biophys Acta* 1758: 264–279.
- Rhodes DR, Chinnaiyan AM (2005). Integrative analysis of the cancer transcriptome. *Nat Genet* 37 (Suppl): S31–S37.
- Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, Chernomordik LV (2005). Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J Biol Chem* 280: 15300–15306.
- Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ *et al.* (2003). Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 278: 585–590.
- Rojas M, Donahue JP, Tan Z, Lin YZ (1998). Genetic engineering of proteins with cell membrane permeability. *Nat Biotechnol* 16: 370–375.
- Saar K, Lindgren M, Hansen M, Eiriksdottir E, Jiang Y, Rosenthal-Aizman K *et al.* (2005). Cell-penetrating peptides: a comparative membrane toxicity study. *Anal Biochem* 345: 55–65.
- Sampath P, Pollard TD (1991). Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. *Biochemistry* 30: 1973–1980.
- Schmid SL (1997). Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu Rev Biochem* 66: 511–548.
- Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF (1999). *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* 285: 1569–1572.
- Schwarze SR, Hruska KA, Dowdy SF (2000). Protein transduction: unrestricted delivery into all cells? *Trends Cell Biol* 10: 290–295.
- Skehel JJ, Cross K, Steinhauer D, Wiley DC (2001). Influenza fusion peptides. *Biochem Soc Trans* 29: 623–626.
- Sugita T, Yoshikawa T, Mukai Y, Yamanada N, Imai S, Nagano K *et al.* (2007). Improved cytosolic translocation and tumor-killing



- activity of Tat-shepherdin conjugates mediated by co-treatment with Tat-fused endosome-disruptive HA2 peptide. *Biochem Biophys Res Commun* 363: 1027–1032.
- Tyagi M, Rusnati M, Presta M, Giacca M (2001). Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *J Biol Chem* 276: 3254–3261.
- Wadia JS, Stan RV, Dowdy SF (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 10: 310–315.
- West MA, Bretscher MS, Watts C (1989). Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J Cell Biol* 109: 2731–2739.
- Yandek LE, Pokorny A, Floren A, Knoelke K, Langel U, Almeida PF (2007). Mechanism of the cell-penetrating peptide transportan 10 permeation of lipid bilayers. *Biophys J* 92: 2434–2444.
- Ziegler A, Seelig J (2004). Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters. *Biophys J* 86: 254–263.

## COMMUNICATION

# Organelle-Targeted Delivery of Biological Macromolecules Using the Protein Transduction Domain: Potential Applications for Peptide Aptamer Delivery into the Nucleus

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Extensive effort is currently being expended on the innovative design and engineering of new molecular carrier systems for the organelle-targeted delivery of biological cargoes (e.g., peptide aptamers or biological proteins) as tools in cell biology and for developing novel therapeutic approaches. Although cell-permeable Tat peptides are useful carriers for delivering biological molecules into the cell, much internalized Tat-fused cargo is trapped within macropinosomes and thus not delivered into organelles. Here, we devised a novel intracellular targeting technique to deliver Tat-fused cargo into the nucleus using an endosome-disruptive peptide (hemagglutinin-2 subunit) and a nuclear localization signal peptide. We show for the first time that Tat-conjugated peptide aptamers can be selectively delivered to the nucleus by using combined hemagglutinin-2 subunit and nuclear localization signal peptides. This nuclear targeting technique resulted in marked enhancement of the cytostatic activity of a Tat-fused p53-derived peptide aptamer against human MDM2 (mouse double minute 2) that inhibits p53–MDM2 binding. Thus, our technique provides a unique methodology for the development of novel therapeutic approaches based on intracellular targeting.

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**Keywords:** Tat; HA2; nuclear localization signal; intracellular targeting; peptide aptamer

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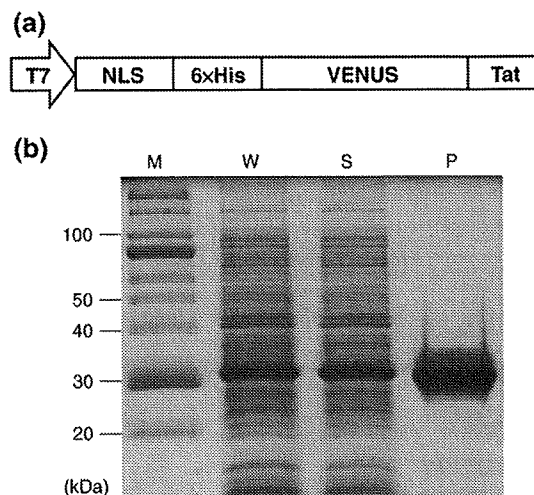
Abbreviations used: MDM2, mouse double minute 2; PTD, protein transduction domain; HA2, hemagglutinin-2 subunit; NLS, nuclear localization signal; NLS–VENUS–Tat, VENUS protein with Tat, NLS and His tag; Tat–cargo, Tat-fused cargo; HA2–Tat, Tat-fused endosome-disruptive HA2 peptide; PM10–Tat, Tat-fused PM10; NLS–PM10–

Progress in molecular biological research on intractable diseases such as cancer is steadily increasing our knowledge of the mechanisms of malignant transformation occurring in tumor cells. However, the complexity of biological interactions makes it increasingly difficult to predict gene and protein functions as we proceed from the immediate metabolic pathway to the levels of the cell and organism. Especially at the cellular level, a variety of tools are available to determine protein function and to develop novel therapeutic approaches, including antisense, peptide modulators, proteins and the overexpression of wild-type or dominant-negative proteins. Small peptides might be able to complement these agents because of

their ability to recognize specific protein domains and thus to interfere with enzymatic functions or protein-protein interactions.<sup>1</sup> Furthermore, these peptides, designated "peptide aptamers," seem to be non-genotoxic and useful for adjunct chemotherapy. However, these approaches are often limited by an inability to effectively deliver the agents to the appropriate cellular location.

Because a prerequisite for their intracellular action is their delivery into cells, various intracellular delivery approaches, such as protein transduction technology with protein transduction domains (PTDs; e.g., Tat, Antp, VP22, Rev and so on), are currently undergoing intensive scrutiny.<sup>2,3</sup> However, protein transduction technology using PTDs has some disadvantages, one of which is the accumulation of PTDs or PTD-fused peptides in the endocytic compartment. We and others have reported that the main mechanism of protein transduction is penetration into cells by macropinocytosis and that therefore much of the material taken up remains entrapped in the macropinosomes.<sup>4-7</sup> Another disadvantage is that there is no technique for controlling the intracellular distribution of peptide aptamers such that their effects are extremely limited. For these reasons, it is important that peptide aptamers be delivered directly into specific cellular compartments. Because optimal approaches for overcoming these disadvantages have not yet been developed, high concentrations of PTDs or PTD-fused peptides must still be employed in order for the technology to function effectively.

With this in mind, we focused on developing intracellular targeting technology for peptide aptamers fused with Tat protein basic domain residues 47-57 (YGRKKRRQRRR) from human immunodeficiency virus-1 in the context of cancer therapeutics. We recently reported that survivin-targeted peptide aptamers (shepherdin) linked to the NH<sub>2</sub>-terminal domain of the influenza virus hemagglutinin-2 subunit (HA2), which is a pH-dependent fusogenic peptide inducing lysis of membranes at low pH levels,<sup>8,9</sup> are efficiently released from macropinosomes. This approach succeeded in inducing the death of survivin-expressing malignant tumor cells.<sup>10</sup> Based on this previous study, here we devised a novel intracellular targeting technology using HA2 and nuclear localization signal (NLS) peptides for converting peptide aptamers into efficient research tools for cell biology and cancer therapeutics. We established for the first time that Tat-conjugated peptide aptamers can be selectively delivered to the nucleus by combining HA2 and NLS peptides. Furthermore, we evaluated the utility of our nuclear targeting method using p53-derived peptide from the human MDM2 (mouse double minute 2, also termed HDM2 in humans)-binding domain (residues 17-26; designated PM10), which is a peptide inhibitor of p53-MDM2 binding.<sup>11</sup> Recent reports suggested that inhibiting p53-MDM2 binding could reactivate the p53 pathway and induce growth-suppressive effects and cell cycle arrest of tumor cells as well as normal



**Fig. 1.** Vector construction and SDS-PAGE analysis of NLS-VENUS-Tat. (a) Schematic of the NLS-VENUS-Tat region of T7 promoter-driven protein expression vector. The VENUS (variant of yellow fluorescent protein) DNA sequence was kindly provided by Dr. A. Miyawaki (RIKEN Brain Science Institute, Saitama, Japan). The NLS-VENUS-Tat DNA sequence was amplified by PCR. At the 5' end, the primer sequence 5'-AA CTT TAA GAA GGA GAT ATA CAT ATG CCG AAA AAG AAA CGT AAA GTT ACC ATG GCT CAC CAC CAT CAC CAC CAT GAC TAC AAA GAC GAT GAT GAC AAA GAA GCT TAC GTG AGC AAG GGC GAG GAG CTG TT-3' introduced an NdeI site (italics), an NLS (boldface) and a 6× His tag (underline); at the 3' end, the primer sequence 5'-T TCC TTT CGG GCT TTG TTA GCA GCC GAA TTC TTA TTA ACG GCG ACG CTG GCG ACG TTT TTT ACG ACC GTA CTC GAG CTT GTA CAG CTC GTC CAT GCC GAG-3' introduced an EcoRI site (italics) and a Tat sequence (boldface). The PCR product was digested with NdeI as well as EcoRI and inserted into pT7 vector, under the control of the T7 promoter. (b) The plasmid was transformed into *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA), and cells expressing VENUS proteins were cultured at 25 °C, 250 rpm, for 6 h. The cell paste was then solubilized in a BugBuster Master Mix (Novagen, Darmstadt, Germany) and centrifuged. NLS-VENUS-Tat was recovered in the supernatant and purified by His-tag affinity purification and gel-filtration chromatography. SDS-PAGE analysis was performed under reducing conditions. Lane M, molecular weight standard; lane W, *E. coli* extracts prepared after induction of expression by IPTG; lane S, soluble fractions; lane P, purified proteins.

cells possessing wild-type p53.<sup>11-17</sup> Because the interaction of p53 and MDM2 takes place inside the nucleus, nuclear delivery of PM10 may potentiate the cytostatic effect of this agent. Indeed, we found that our nuclear targeting technique employing PTD, HA2 and NLS peptides markedly enhanced PM10-mediated cytostatic effects against A549 (human lung adenocarcinoma) and WI-38 (human embryonic fibroblast, lung-derived cell line) cells. These results indicate that our intracellular targeting techniques can deliver the cargo into the appropriate organelle and provide a unique research tool for cellular biology and the development of novel therapeutic approaches.

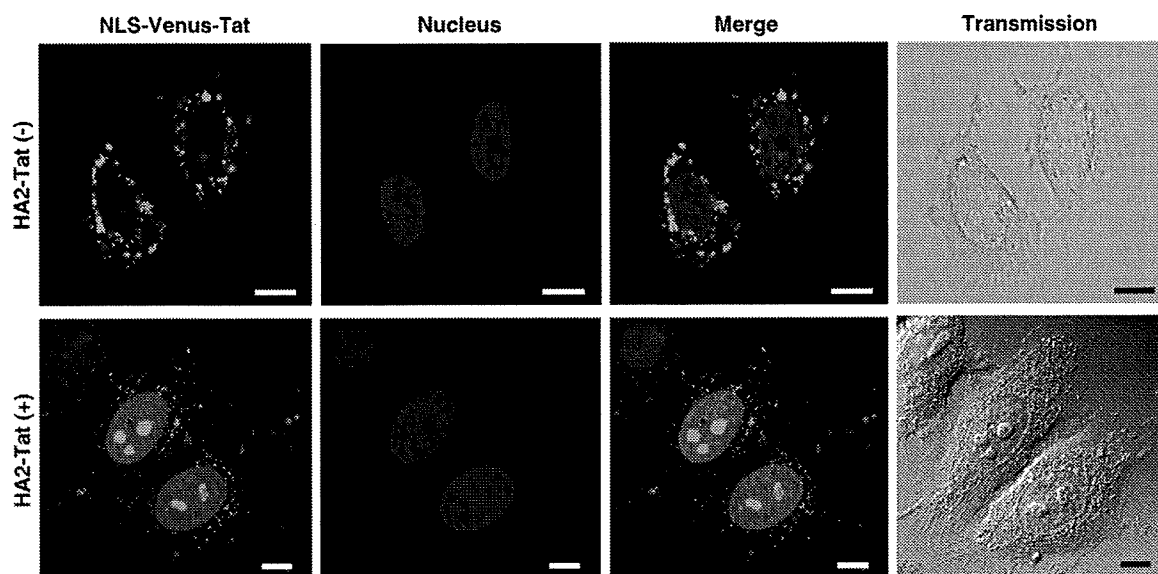
### Tat-fused cargo can be selectively delivered to the nucleus by combining HA2 and NLS peptides

We constructed a VENUS protein (variant of yellow fluorescent protein) fused with Tat, NLS and His tag (NLS-VENUS-Tat) for use as an expression vector (Fig. 1a). NLS-VENUS-Tat was indeed expressed in *Escherichia coli* [BL21 Star (DE3)] after induction with IPTG. The level of expression of the NLS-VENUS-Tat protein was analyzed by SDS-PAGE in total cell lysates (Fig. 1b). Protein expression was specifically induced because we did not find substantially leaky expression of the recombinant protein (data not shown). Recombinant NLS-VENUS-Tat was produced almost entirely in the soluble fraction and had an apparent molecular mass of about 32 kDa under reducing conditions (Fig. 1b, lane S). Purification was carried out by lysis, and separation of the soluble fraction was carried out by centrifugation. This was then loaded onto a Ni<sup>2+</sup> column for initial purification. The NLS-VENUS-Tat protein eluted from the Ni<sup>2+</sup> column was more than 90% pure (Fig. 1b, lane P). The purity, apparent molecular mass and cellular internalization activity of the eluted NLS-VENUS-Tat proteins were established by SDS-PAGE and flow cytometry.

Numerous mechanistic studies have shown that Tat peptides rapidly permeate plasma membranes and translocate into the nucleus.<sup>18–22</sup> This mechanism is currently used to deliver proteins and nucleic acids to cell nuclei through covalent linkages of Tat and cargoes.<sup>18</sup> However, nuclear delivery has remained problematic and very limited, because endosome

escape and nuclear transport of Tat-fused cargo (Tat-cargo) represent a passive rather than an active process. Indeed, in HeLa cells treated with NLS-VENUS-Tat alone, only punctate cytoplasmic fluorescence, and no fluorescence in the nucleus, was observed (Fig. 2). We had previously confirmed that Tat-fused VENUS co-localized in live cells to vesicles with FM4-64, which is a general endosome marker (data not shown). Furthermore, FAM (carboxyfluorescein) dye-fused Tat peptides also co-localized in FM4-64-positive endosomal vesicles (data not shown). Thus, these results indicated that much of the NLS-VENUS-Tat was entrapped within the endosomal vesicles, resulting in low levels of nuclear accumulation. It is therefore reasonable to propose that Tat-cargo alone cannot reach the targeted cellular compartment, especially the nucleus, and that the therapeutic effects of anticancer peptide aptamers are extremely limited for this reason. Therefore, we devised an active nuclear targeting technique using endosome-disruptive HA2 and SV40-derived NLS peptides.

Recently, we reported that co-treatment with Tat-cargo and the Tat-fused endosome-disruptive HA2 peptide (HA2-Tat) improved the endosome-escape ability and the tumor-killing activity of Tat-fused antisurvivin peptide aptamers.<sup>10</sup> Therefore, we hypothesized that NLS-VENUS-Tat can be delivered into the nucleus if NLS-VENUS-Tat can be engineered to escape from the endosomal vesicles into the cytosol by co-treatment with HA2-Tat. To investigate whether co-treatment with HA2-Tat does effectively improve the nuclear localization of NLS-Tat-cargo, we co-treated HeLa cells with NLS-VENUS-Tat and HA2-Tat and analyzed the intracellular localization of NLS-VENUS-Tat by confocal laser scanning



**Fig. 2.** Intracellular distribution of NLS-VENUS-Tat. HeLa cells were cultured on a Lab-Tek II Chambered Coverglass system (Nalge Nunc International) at  $3.0 \times 10^4$  cells/well in MEM (minimum essential medium)- $\alpha$  supplemented with 10% fetal bovine serum and incubated for 24 h at 37 °C. Internalization of NLS-VENUS-Tat was performed as follows: HeLa cells were co-treated with NLS-VENUS-Tat (10  $\mu$ M) with or without HA2-Tat (5  $\mu$ M) in Opti-MEM I (Invitrogen) containing 100 ng/ml of Hoechst 33342 (Invitrogen). After incubation at 37 °C for 3 h, the medium was changed for a fresh medium and assessed by confocal laser scanning microscopy (Leica Microsystems GmbH, Wetzlar, Germany) without cell fixation. Scale bars in each photomicrograph represent 10  $\mu$ m.