

FIGURE 4. TNFR1-selective antagonistic properties of R1antTNF. Different concentrations of wtTNF and R1antTNF were premixed with a fixed concentration of wtTNF-FLAG and were added to the hTNFR1-coated (A) or hTNFR2-coated plates (B). Binding of wtTNF-FLAG was determined as described under "Experimental Procedures." C, to determine the receptor-type contribution to bioactivity, serially diluted R1antTNF was mixed with the human wtTNF (PC60-R1, 200 ng/ml; PC60-R2, 40 ng/ml), and added to the PC60-Rs cells. After 24 h, production of the rat GM-CSF was measured by ELISA as described under "Experimental Procedures." ♦, PC60-R1; 224, PC60-R2. D, Jurkat cells were incubated with the anti-human Fas IgM (0.2 ng/ml) and indicated dilutions of R1antTNF for 24 h, and cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

performed using PC60-R1 and PC60-R2 cell lines that stably expressed hTNFR1 and hTNFR2 (25), respectively. R1antTNF efficiently inhibited the wtTNF-induced GM-CSF production in the PC60-R1 cells but not in the PC60-R2 cells (Fig. 4C), confirming the TNFR1-specific antagonistic activity. The risk of cross-activity to other TNF receptor superfamily members was assessed by Fas-induced cytotoxicity assay on Jurkat cell (Fig. 4D). R1antTNF did not affect the Fas-mediated signaling, which suggest that R1antTNF was highly selective proteo-antagonist of TNFR1. These data suggest that the R1antTNF not only binds to the TNFR1 selectively but also has TNFR1-selective inhibitory activity.

Therapeutic Efficacy of R1antTNF on Lethal Hepatitis Model—To assess the inhibitory activity and therapeutic effect of R1antTNF *in vivo*, we investigated the protective effect of R1antTNF in the TNF/D-(+)-galactosamine (GalN)-induced hepatitis model. GalN is a hepatotoxin that inhibits transcription and translation in hepatocytes. The combined administration of GalN and TNF causes massive apoptosis of hepatocytes and induces lethal hepatitis (36). In the control group, all mice died within 12 h after a lethal challenge with TNF/GalN (Fig. 5A). Co-treatment with R1antTNF improved the survival rate. Especially, in mice co-treated with R1antTNF (270 μ g/mouse), the survival rates were 5/6 after 12 h and 3/6 after 24 h (Fig. 5A). The surviving mice were still alive several weeks after the treatment. In the control group, the serum levels of ALT, a marker for liver dam-

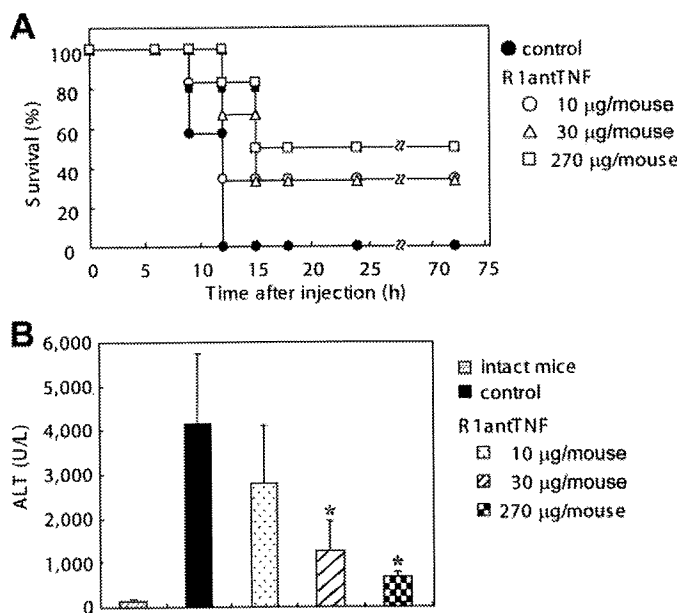


FIGURE 5. Therapeutic effect of R1antTNF in lethal hepatitis model. Mice were injected intravenously with recombinant human TNF (1.0 μ g)/GalN (20 mg) and R1antTNF or PBS. A, survival rates of the mice in the TNF/GalN-induced hepatitis model were measured over a period of 72 h ($n = 6$). B, blood samples were collected 9 h after the challenge. Serum concentration of alanine aminotransferase (ALT) was measured as described under "Experimental Procedures" ($n = 6$). Data represent the mean \pm S.E. Statistical significance versus control mice was calculated by unpaired Student's *t* test (*, $p < 0.05$).

TABLE 3
Binding properties of R1antTNF

Receptor	Kinetic parameter	wtTNF	R1antTNF
hTNFR1	k_{on} ($\times 10^5 M^{-1} s^{-1}$) ^a	3.6 \pm 2.9	8.3 \pm 0.1
	k_{off} ($\times 10^{-4} s^{-1}$) ^b	5.0 \pm 1.3	28.7 \pm 5.9
	K_D ($\times 10^{-9} M$) ^c	1.4 \pm 2.7	3.5 \pm 1.8
hTNFR2	k_{on} ($\times 10^5 M^{-1} s^{-1}$)	7.0 \pm 3.2	0.000001 \pm 0.0
	k_{off} ($\times 10^{-4} s^{-1}$)	14.5 \pm 4.9	0.1 \pm 0.0
	K_D ($\times 10^{-9} M$)	2.1 \pm 1.1	92,900 \pm 96.1
mTNFR1	k_{on} ($\times 10^5 M^{-1} s^{-1}$)	2.5 \pm 0.0	18.7 \pm 0.2
	k_{off} ($\times 10^{-4} s^{-1}$)	1.9 \pm 0.3	96.6 \pm 0.8
	K_D ($\times 10^{-9} M$)	0.8 \pm 0.4	5.2 \pm 0.5

^a k_{on} is the association kinetic constant.

^b k_{off} is the dissociation kinetic constant.

^c K_D is the equilibrium dissociation constant. Kinetic parameters for each TNF was calculated from the respective sensorgram by BIAevaluation 3.0 software.

age, were markedly elevated. In contrast, co-treatment of mice with R1antTNF suppressed the elevation of ALT levels in a dose-dependent manner (Fig. 5B). These results demonstrated that R1antTNF had antagonistic activity not only *in vitro* but also *in vivo*, and exhibited remarkable inhibitory effect on hepatitis.

Binding Mode and Affinity of R1antTNF—The dissociation constant (K_D) of R1antTNF binding to hTNFR1 was very similar to that of the wtTNF (Table 2), whereas the R1antTNF (mutTNF-T2) has no bioactivity through TNFR1 (Fig. 2A). We predicted that the binding modes of the wtTNF and R1antTNF to the TNFR1 are different, resulting in different biological activities. To quantify the altered binding mode of R1antTNF, we examined the binding kinetics of the R1antTNF for the mTNFR1, hTNFR1, and hTNFR2 using the surface plasmon resonance technique (Table 3). Indeed, the dissociation kinetic constants (k_{off}) of the R1antTNF for the human and mouse

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TNFR1 (hTNFR1, $16.0 \times 10^{-4} \text{ s}^{-1}$; mTNFR1, $37.0 \times 10^{-4} \text{ s}^{-1}$) were clearly higher than those of the wtTNF (hTNFR1, $3.0 \times 10^{-4} \text{ s}^{-1}$; mTNFR1, $1.5 \times 10^{-4} \text{ s}^{-1}$). The association kinetic constants (k_{on}) of the R1antTNF for the human and mouse TNFR1 were also higher than those of the wtTNF. These results suggest that the R1antTNF interacts with the TNFR1 by repeating very quick binding and dissociation, and has a binding mode that is different from that of the wtTNF.

TABLE 4
X-ray data collection and refinement statistics (molecular replacement)

Crystal	R1antTNF
Data collection	
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	66.56, 66.97, 103.56
Resolution (Å)	50.0-1.80 (1.86-1.80) ^a
<i>R</i> _{merge}	0.063 (0.484)
<i>I</i> / <i>σI</i>	36.8 (2.71)
Completeness (%)	99.6 (96.1)
Redundancy	7.1 (6.0)
Refinement	
Resolution (Å)	41.0-1.80
No. of reflections	42,155
<i>R</i> _{work} / <i>R</i> _{free}	19.8/23.9
No. of atoms	
Protein	3384
Water	237
Root mean squares deviations	
Bond lengths (Å)	0.00840
Bond angles (°)	1.47

^a Highest resolution shell is shown in parentheses.

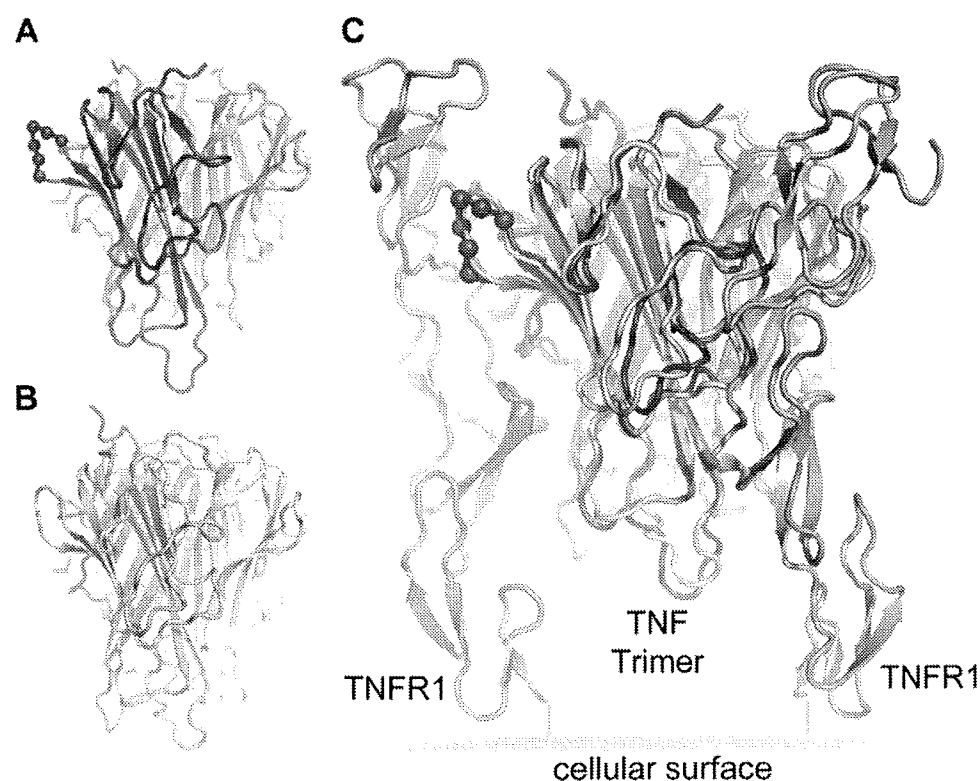


FIGURE 6. Overall structures of R1antTNF and wtTNF. A, refined structure of the R1antTNF trimer (green). Blue spheres show the mutated residues (amino acids 84–89) in R1antTNF. This structure is registered in the PDB (PDB code 2E7A). B, structure of the wtTNF trimer (gray). This structure has been published, and its PDB code is 1TNF. C, model structures of the TNF-TNFR1 complexes. Each TNF is superposed on the LT- α derived from the LT- α -TNFR1 complex (PDB code 1TNR). TNF binds to three R1 monomers on the cell surface. TNFR1s are shown using red schematics. Superposition of the structures of the wtTNF and R1antTNF was performed using the ccp4i program.

Crystal Structure of R1antTNF—To understand the structural basis for the different binding mode and absence of signal transduction via the R1antTNF, we examined the structure of R1antTNF by x-ray crystallography. After establishing crystallization conditions, good quality crystals of R1antTNF were obtained. The R1antTNF crystal size was $\sim 0.2 \times 0.2 \times 0.4 \text{ mm}^3$. X-ray diffraction data were collected in SPring-8 (the large synchrotron radiation facility in Japan). Analysis of these data show that the space group is P2₁2₁2₁, and the lattice constants are *a* = 64.56, *b* = 66.97, and *c* = 103.56 Å (Table 4). The R1antTNF structure was further refined using CNS. Results of the model validation using the Procheck were as follows: 88.4% residues in the most favored regions; 11.0% residues in the additional allowed regions; 0.6% residues in the generously allowed regions; and 0.0% residues in the disallowed regions. The overall structure of the R1antTNF was a trimer (Fig. 6A) (PDB code 2E7A), which was similar to that of the wtTNF trimer (Fig. 6B) (PDB code 1TNF) (37). Interestingly, structural superposition of the R1antTNF and human wtTNF showed extraordinary similarity (root mean square deviation 1.17 Å for 444 C- α atoms) of their overall structures despite their contradictory functions (Fig. 6C). It is believed that the TNF signaling is initiated by the formation of a complex with the three TNFRs on the cell surface. However, the fact that the R1antTNF did not transduce signaling suggests that there might be other structural differences between the wtTNF and R1antTNF.

DISCUSSION

TNF, secreted from the site of injury or because of the activation of the immune cells, is involved in the development of inflammatory diseases, and it predominantly activates TNFR1 (40, 41). The TNFR1 knock-out mice have been reported to be resistant to the onset of several inflammatory diseases, such as sepsis, rheumatoid arthritis, and multiple sclerosis (14, 16, 42). In agreement with this, blocking the interaction between the TNF and TNFR1 has emerged as a powerful and clinically effective therapy for the acute inflammation and autoimmune conditions. In this study, we generated TNFR1-selective antagonistic TNF mutants using a phage library displaying structural variants of human TNF.

Among 10 potential candidates, the mutTNF-T2 (R1antTNF) selectively and strongly bound to the TNFR1 but showed almost no bioactivity. Additionally, we found that R1antTNF most effectively inhibited the wtTNF-induced cell death

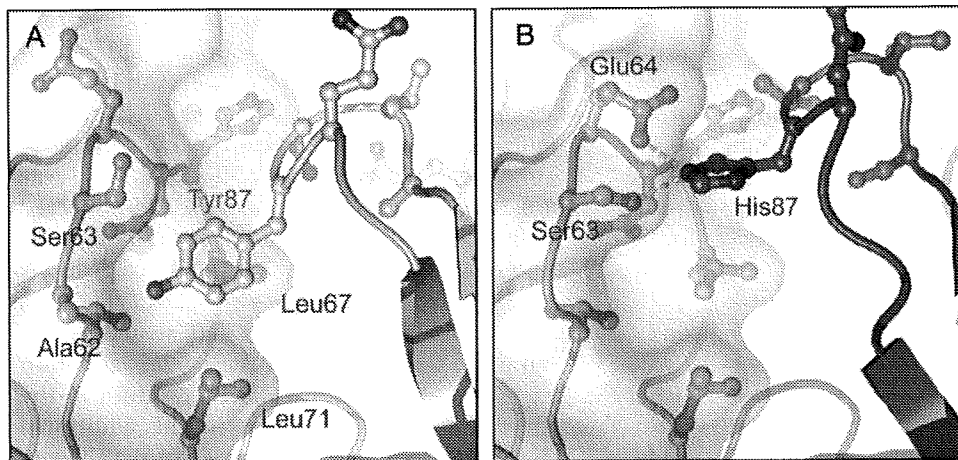


FIGURE 7. Structural difference between the receptor binding region of the R1antTNF and wtTNF. A, interaction between the wtTNF (gray) and TNFR1 (red). White layer depicts the molecular surface of the TNFR1. Hydrophobic interaction is formed between the Tyr-87 and molecular pocket in the TNFR1 (Leu-67, Leu-71, Ala-62, and Ser-63). B, interaction between the R1antTNF (green) and TNFR1 (red). Yellow broken lines show the possible interactions of the R1antTNF His-87 with the receptor Ser-63 and Glu-64. In this simulation, the side chains of each structure were rotated to fit the predicted interaction. Stable structures of these rotamers were constructed using the O program.

(Fig. 3B). R1antTNF also clearly inhibited the TNF functions other than cytotoxicity. Interestingly, the ratio between the R1antTNF and wtTNF was 1000-fold or more to obtain 50% inhibition against the wtTNF-induced cytotoxicity and caspase-3/7 activity, whereas the ratio was only about 100-fold or less to obtain 50% inhibition against the wtTNF-induced expression of E-selectin, production of GM-CSF, and NF- κ B activation. Intracellular signaling induced by the TNF/TNFR1 interaction is divided into the following two main pathways: (i) the NF- κ B pathway, which regulates the expression of adhesion molecules and inflammatory cytokines, and (ii) the caspase cascade, which induces cell death through apoptosis or necrosis (34). Our results suggest that R1antTNF is more antagonistic against the wtTNF function mediated via the NF- κ B pathway than via the caspase cascade. In pathological tissues, endogenous TNF induced expression of the cell adhesion molecules and inflammatory cytokines resulting in leukocyte infiltration, which are regulated by NF- κ B and are closely related to the development or exacerbation of diseases (43) such as fulminant hepatitis and rheumatoid arthritis. Because the R1antTNF efficiently inhibited the TNF-induced NF- κ B activation, it would be of therapeutic value for the treatment of such inflammatory diseases. These cell line or signaling pathway-dependent differences in the inhibitory efficiencies of R1antTNF could be valuable in studying the structural or biological changes caused by the TNF/TNFR1 interactions, which need to be explored further.

We examined the therapeutic effects of R1antTNF in the acute lethal hepatitis model. R1antTNF exhibited the suppressive effect on acute hepatitis. Although the R1antTNF suppressed the elevation of the ALT level in a dose-dependent manner, the survival rate was not significantly improved between the dose 10 μ g/mouse versus 270 μ g/mouse. This discrepancy is likely because the difference in the degree of suppression of the elevated ALT level does not always correlate well with the improvement in the survival rate. Nevertheless, treatment with only 30–270 μ g/mouse R1antTNF (30–270-

fold excess over wtTNF) significantly suppressed the elevation of ALT levels and reduced the lethal toxicity. Thus, the antagonistic activity of R1antTNF *in vivo* was stronger than expected from the *in vitro* results. The TNF/GalN-mediated activation of TNFR1 not only induces apoptosis and necrosis of hepatocytes but also induces inflammatory responses and secondary responses associated with the cell death (44). Therefore, we believe that the R1antTNF exerted its therapeutic effect by comprehensively inhibiting the TNF/GalN-mediated biological responses, thus blocking the liver failure in the experimental animals. The R1antTNF is also expected to have therapeutic effect

in chronic inflammatory disease models, such as in collagen-induced arthritis model and experimental autoimmune encephalomyelitis model. However, the plasma half-life of R1antTNF, like the wtTNF, is very short (12 min). We recently developed a novel PEGylation system that dramatically improved the *in vivo* stability and therapeutic effects of the bioactive proteins (29, 45). We are currently in the process of developing the PEGylated R1antTNF to further enhance its potential anti-inflammatory activity.

To explore the underlying mechanism of the antagonistic activity of R1antTNF, we examined the crystal structure of R1antTNF by x-ray crystallography. Despite close resemblance between the crystal structure of the R1antTNF and wtTNF, the receptor-bound R1antTNF did not transmit any signal via the TNFR1. To further speculate why the R1antTNF showed antagonistic activity, we utilized the superpose program to perform docking simulations with the TNFs and TNFR1 using the crystal structure of the LT- α -TNFR1 complex (PDB code 1TNR) (31). The TNF-TNFR1 model complex suggested that the Tyr-87 of the wtTNF, an essential residue, is buried in a molecular hydrophobic "pocket" of the TNFR1, which houses the receptor residues Leu-67 and Leu-71 that are implicated to maintain the TNF and TNFR1 complex (Fig. 7A). Tyr-87 is a highly conserved residue throughout the TNF superfamily, such as LT- α , LT- β and LIGHT, and this discussion is also reflected in the crystal structure of the LT- α -TNFR1 complex (38). Accordingly, site-directed mutagenesis of the Tyr-87 residue of TNF resulted in a dramatic loss of its biological activity and affinities for both TNFRs (39), suggesting that this residue is essential for TNF function. However, in R1antTNF, the Tyr-87 is replaced with a histidine residue. The structural simulation studies suggested that the His-87 in R1antTNF could interact with the relatively negatively charged Ser-63 and Glu-64 residues on the TNFR1 surface, which probably explains the different binding mode of the R1antTNF as compared with the wtTNF (Fig. 7B).

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Indeed, the association and dissociation kinetic constants (k_{on} and k_{off} respectively) for the binding of R1antTNF to TNFR1 were considerably higher than those of the wtTNF, indicating a difference in the TNFR1-binding pattern between the wtTNF and R1antTNF (Table 3). The association and dissociation kinetic constants are very important factors in discussing the ligand-receptor interaction and function. It was previously shown that the membrane-bound TNF, but not the soluble TNF, could activate the TNFR2, and the reason for this difference was attributed to the dissociation kinetic constant of the soluble TNF, which was much higher than the membrane-bound TNF (46). Therefore, the inability of R1antTNF to transmit signal and its antagonistic activity are probably because of the higher k_{on} and k_{off} values of R1antTNF for the TNFR1, suggesting that they influenced the stability of the TNF-TNFR1 complex and reduced the continuous binding time required for the signal transmission. In addition, we demonstrated that R1antTNF inhibited the activations of both caspase-3/7 and NF- κ B (Fig. 5, A and B), which are mediated via two distinct intracellular signaling complexes. TNF/TNFR1-mediated signaling requires sequential formation of the following two receptor complexes: the complex I is involved in the recruitment of TRADD, RIP1, and TRAF2 leading to the NF- κ B activation, whereas the consequent complex II is involved in internalization, post-translational modifications, and recruitment of FADD and caspase-8 initiating apoptosis (47). Together, these results suggest that R1antTNF blocked the TNF-mediated signal transmission by binding to TNFR1 in a rapid association/dissociation cycle, thereby inhibiting the formation of the intracellular complexes. However, more detailed investigations, such as detection of these intracellular complexes and their internalization, other TNF/TNFR1-mediated signaling, and analysis of the complex structure of the R1antTNF and TNFR1, are required to elucidate exactly how the R1antTNF exhibits its TNFR1 antagonistic activity.

We succeeded in developing the first mutant form of the human TNF with TNFR1-selective antagonistic activity by using a unique combinatorial phage-based technique. Existing TNF blockers, *i.e.* etanercept and infliximab, are widely used in the treatment of rheumatoid arthritis and Crohn disease (5). But these drugs, which prevent TNF binding on both TNF receptor types, can cause serious side effects, such as mycobacterial infections and hepatitis B virus infection (48). Although TNFR1 is believed to be important for immunological responses (42), TNFR2 is thought to be also important for antiviral resistance and is effective for controlling the mycobacterial infection by affecting the membrane-bound TNF stimulation (18, 49). Therefore, this mutant TNF, R1antTNF, might be a new therapeutic drug with reduced side effects. We are currently evaluating not only the therapeutic effect of R1antTNF on rheumatoid arthritis or experimental encephalomyelitis model, and but also its side effects such as mycobacterial and virus infection. Finally, our studies demonstrate the feasibility of generating TNF receptor subtype-selective antagonistic mutants by comprehensive substitution of sets of amino acids in the wild-type ligand proteins. Our data also suggest that this combinatorial biosynthetic strategy using the bioactive pro-

tein as the "lead protein" is very effective in creating receptor-specific agonists and antagonists, and we believe that this approach will generate protein drugs of improved therapeutic value.

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RESEARCH PAPER

Comparative study on transduction and toxicity of protein transduction domains

T Sugita^{1,2}, T Yoshikawa¹, Y Mukai^{1,2}, N Yamanada^{1,2}, S Imai^{1,2}, K Nagano^{1,2}, Y Yoshida^{1,3}, H Shibata¹, Y Yoshioka^{1,4}, S Nakagawa², H Kamada^{1,4}, S-i Tsunoda^{1,4} and Y Tsutsumi^{1,3,4}

¹Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation (NIBIO), Ibaraki, Osaka, Japan;

²Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan;

³Department of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan and ⁴The Center for Advanced Medical Engineering and Informatics, Osaka University, Suita, Osaka, Japan

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- β -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,

Correspondence: Dr S-i Tsunoda, Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation (NIBIO), 7-6-8 Saito-Asagi, Ibaraki, Osaka 597-0085, Japan.

E-mail: tsunoda@nibio.go.jp

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followed by the association of these complexes with lipid rafts, which triggers dynamin-independent macropinoscytosis. 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 α ; 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	<i>pI</i>
Tat	HIV-1	GRKKRRQRRRPPQ	12.70
Antp	<i>Drosophila</i>	RQIKIWFAQNRRMKWKK	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% CO₂.

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), TRQARRNRRRRWRERQRK-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, TQRARRNRRRRWRERQRK-biotin for Rev-conjugated biotin, NAKTRRHERRRKLAIERK-biotin for VP22-conjugated biotin and GLFEAIEGFIENGWEGMIDGWY GYGRKKRRQRRR 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 5.0×10^4 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 1.0×10^5 cells per well in Opti-MEM I (Invitrogen, Carlsbad, CA, USA). After aspirating the media, FAM-conjugated PTD (PTD-FAM) (10 μ 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 μ 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- β -cyclodextrin, 0–2.5 μ 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 5.0×10^3 cells per well in MEM α and incubated for 24 h at 37 °C. Jurkat cells were cultured in 96-well plates at 1.0×10^4 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 5.0×10^3 cells per well in MEM α and incubated for 24 h at 37 °C. Jurkat cells were cultured in 96-well plates at 1.0×10^4 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 TGTTACAC-3' introduced an *Nde* I site and Tat sequence and at the 3' end, the primer sequence 5'-GCTTTGTTAGCAG CCGAATTCTTACTTGTACAGCTCGTCCATGCCGAGAGTGA 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 GAATCGTCGTATGAAGTGGAAAAAAGGTGGCGGCGGTTCC-3'; Antp antisense, 5'-TCGAGGGAACCGCCGCCACCTT TTTTCCACTTCATACGACGATTCTGGAACCAGATTTTGATC TGACGAGCCA-3'; Rev sense, 5'-TATGGCTACCCGTCAGGC TCGTCGTAATCGTCGTCGTCGTTGGCGTGAACGTCAGCGT GGTGGCGGCGGTTCCC-3'; Rev antisense, 5'-TCGAGGGAA CCGCCGCCACCACGCTGACGTTACGCCAACGACGACGA CGATTACGACGAGCCTGACGGGTAGCCA-3'; VP22 sense, 5'-TATGGCTAACGCTAAAACCCGTCGTCACGAACGTCGTCG TAAACTGGCTATCGAACGTGGTGGCGGCGGTTCCC-3'; VP22 antisense, 5'-TCGAGGGAACCGCCGCCACCACGTTTCGATAG CCAGTTTACGACGACGTTTCGTCGACGACGCGGTTTTCGCGT 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 Coverglass (Nalge Nunc International) at 3.0×10^4 cells per well in MEM α 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 μ M) in Opti-MEM I containing 100 ng ml⁻¹ Hoechst 33342 (Invitrogen) and 6 μ g ml⁻¹ 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 μ M) and HA2-Tat (2 μ M) in Opti MEM I containing 100 ng ml⁻¹ 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

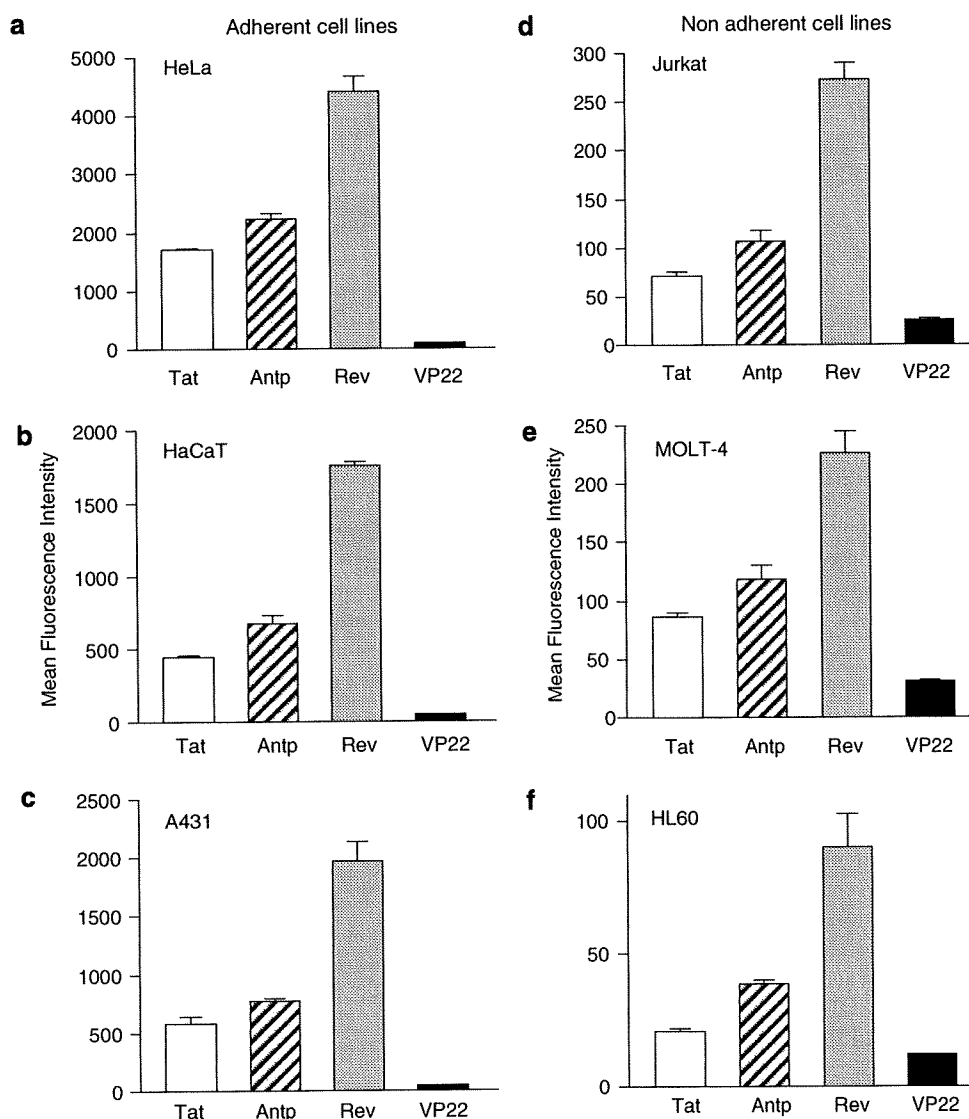


Figure 1 Comparison of the cellular uptake of protein transduction domains (PTDs). FAM-labelled Tat (white column), antennapedia (Antp; hatched column), Rev (grey column) and VP22 (black column) were incubated with six cell lines: HeLa (a), HaCaT (b), A431 (c), Jurkat (d), MOLT-4 (e) and HL60 (f) at $10\ \mu\text{M}$ 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

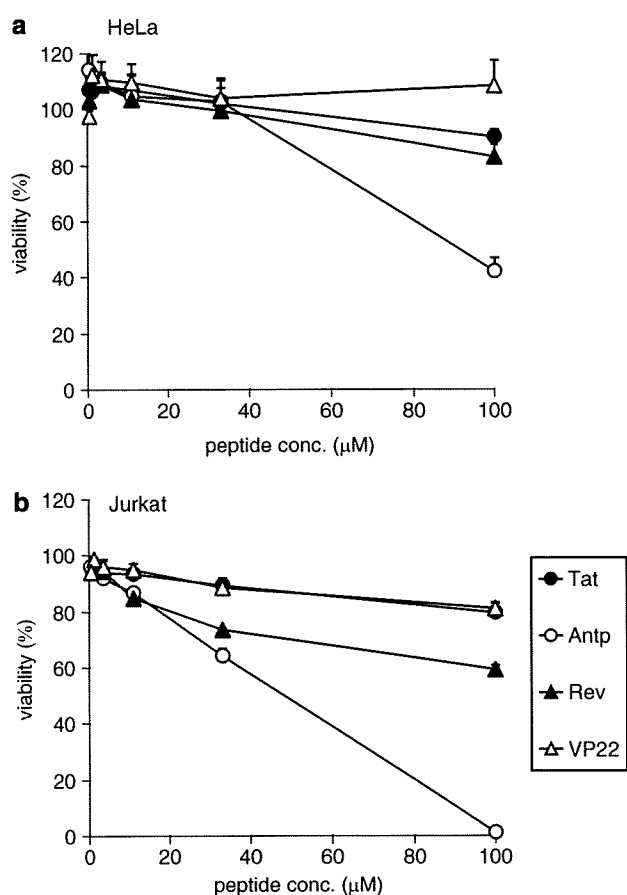


Figure 2 Viability of protein transduction domain (PTD)-treated cells. HeLa cells (a) and Jurkat cells (b) were incubated with serially diluted biotin-conjugated Tat, antennapedia (Antp), Rev and VP22 at 37 °C. After 24 h, cell viability was analysed using a WST-8 assay. Data shown are the mean \pm s.d.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

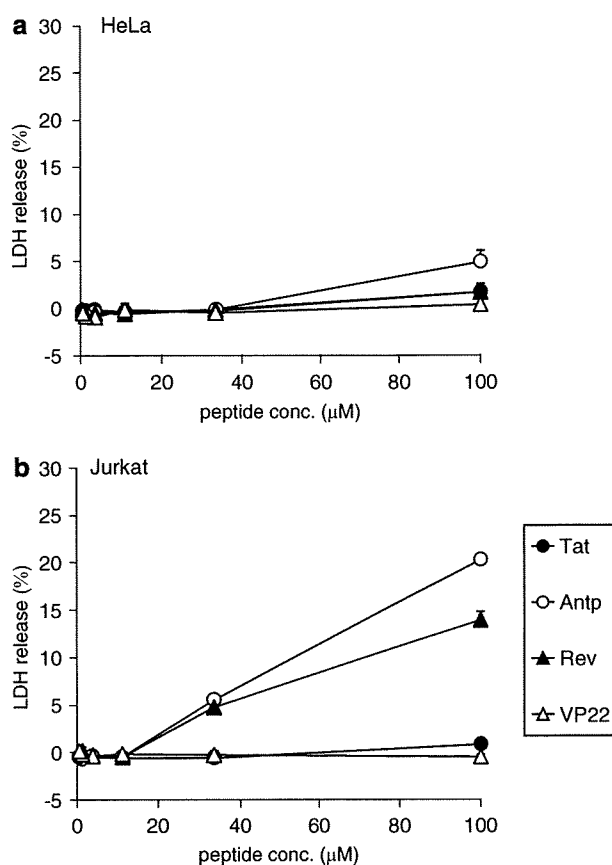


Figure 3 Membrane integrity of protein transduction domain (PTD)-treated cells. HeLa cells (a) and Jurkat cells (b) were incubated with serially diluted biotin-conjugated Tat, antennapedia (Antp), Rev and VP22 at 37 °C. After 3 h, the release of lactate dehydrogenase (LDH) was analysed. Data shown are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

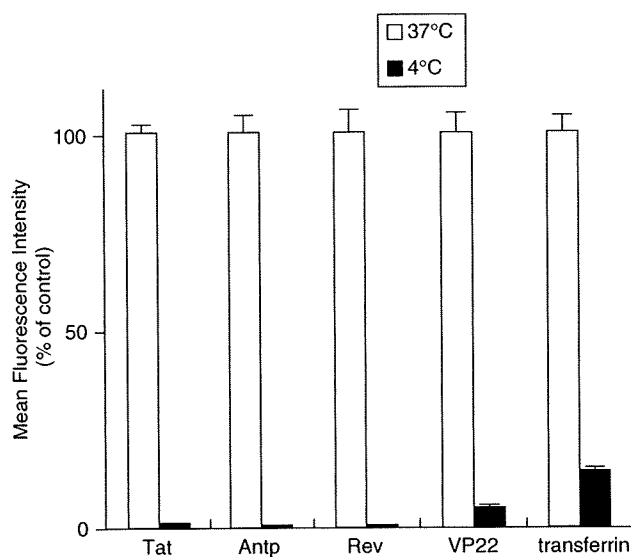


Figure 4 Effects of temperature on protein transduction domain (PTD) transduction efficiency. HeLa cells were preincubated at 37 or 4°C for 1 h prior to adding FAM-labelled PTDs or fluorescein isothiocyanate-labelled transferrin for 3 h. Cells were washed in trypsin and analysed by flow cytometry. Data 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

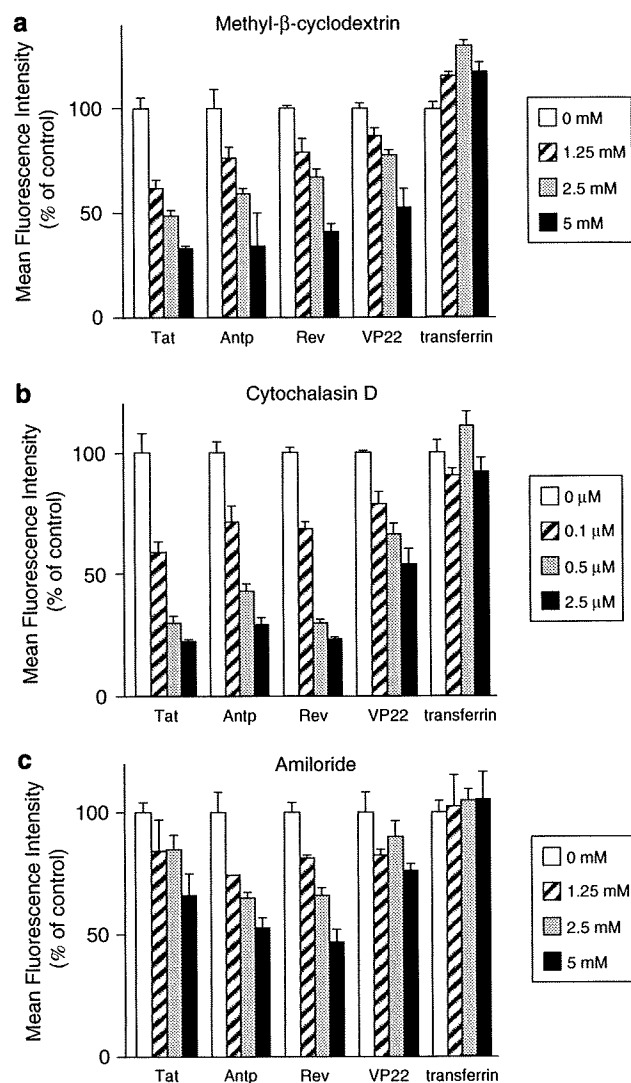


Figure 5 Effects of endocytosis inhibitors on transduction efficiency of protein transduction domains (PTDs). HeLa cells were pretreated with a range of concentrations of (a) methyl-β-cyclodextrin, (b) cytochalasin D or (c) amiloride for 30 min prior to adding FAM-labelled PTDs or fluorescein isothiocyanate-labelled 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,

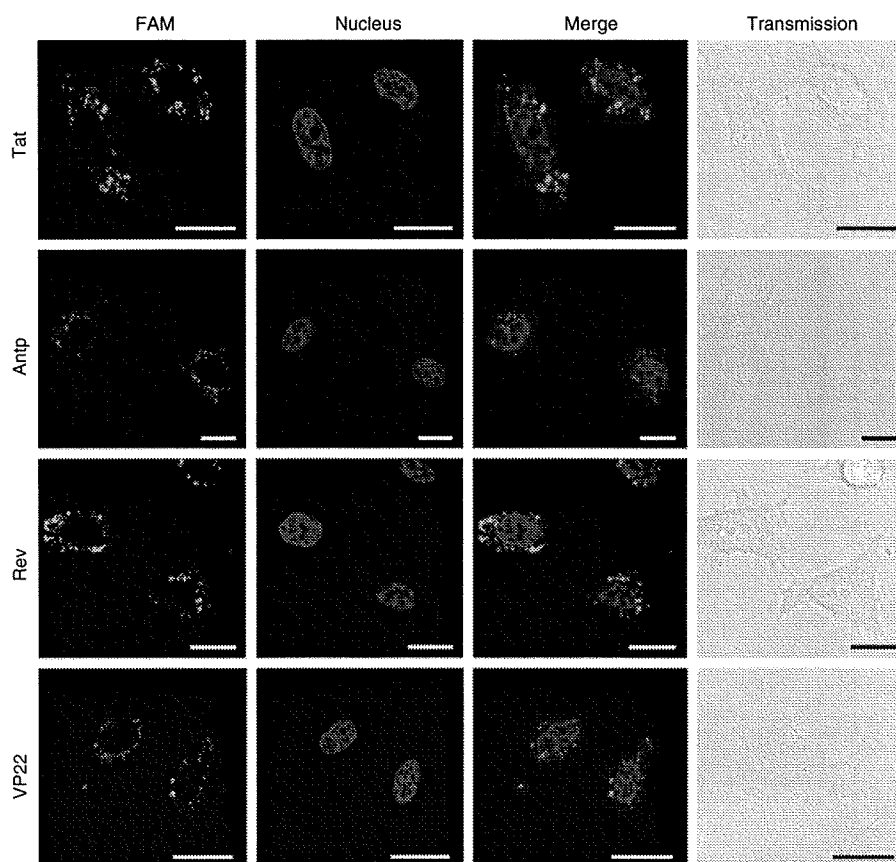


Figure 6 Intracellular behaviour of protein transduction domain (PTD)-FAM in living cells. HeLa cells were treated with 10 μ M PTD-FAM for 3 h. Fluorescence images were acquired using confocal laser scanning microscopy and the signals were merged electronically. The nucleus was counterstained with Hoechst 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 μ 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,

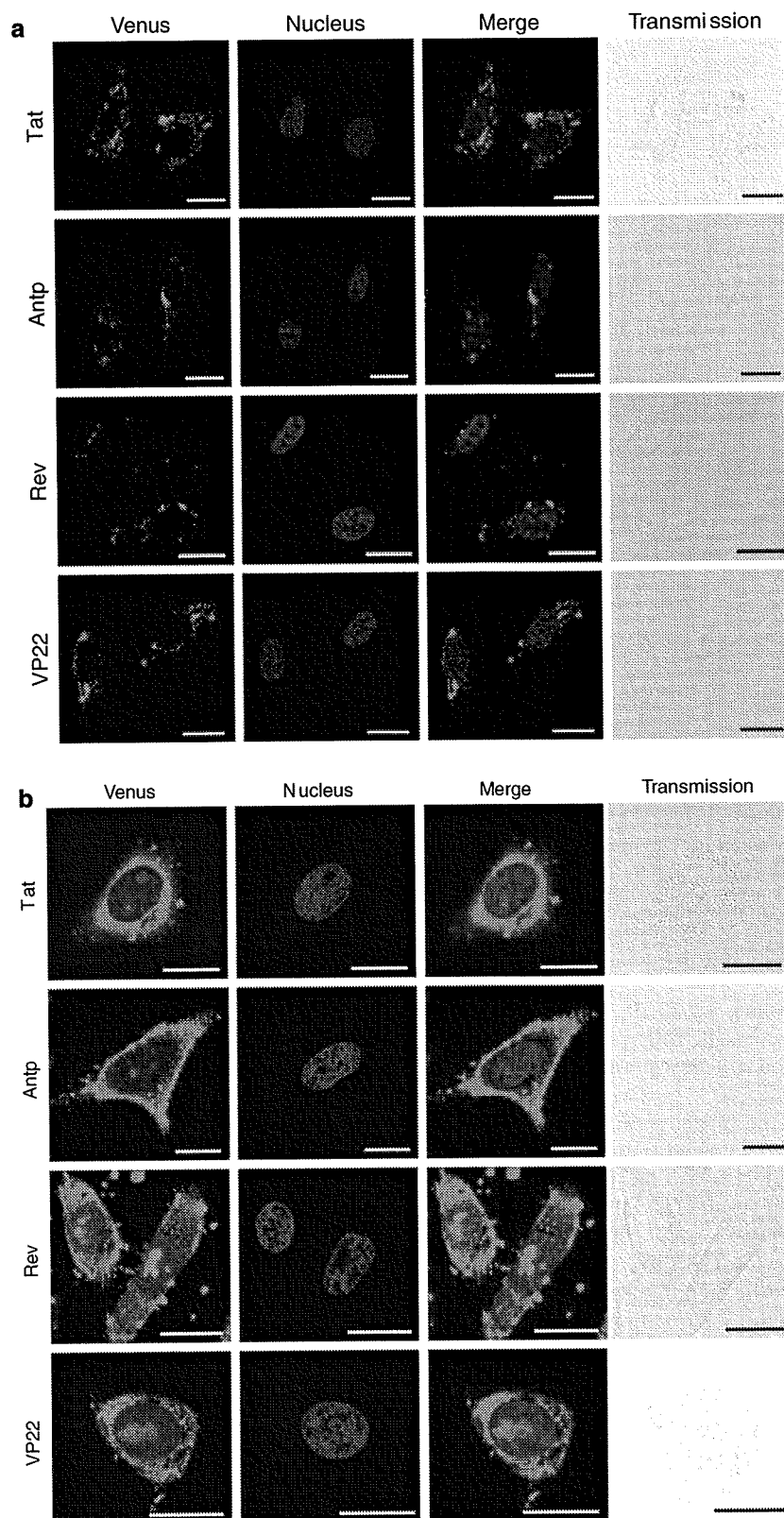


Figure 7 Intracellular behaviour of protein transduction domain (PTD)-Venus in living cells. HeLa cells were treated with 10 μM PTD-Venus alone (a) or 10 μM HA2-Tat (b) for 3 h. Fluorescence images were acquired using confocal laser scanning microscopy and the signals were merged electronically. The 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 μm.

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.

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Mini Review

Development of new anti-TNF therapy

Haruhiko Kamada *, Hiroko Shibata, and Yasuo Tsutsumi

Laboratory of Pharmaceutical Proteomics (LPP) National Institute of Biomedical Innovation (NIBIO), Osaka, Japan

We have generated the first TNFR1-selective antagonistic TNF mutant based on structural human TNF variants using our phage display technology. This TNF mutant did not activate TNFR1-mediated responses, although its affinity for TNFR1 was equivalent to human wild-type TNF (wtTNF). The TNF mutant neutralized wtTNF-induced TNFR1-mediated bioactivity without influencing TNFR2-mediated bioactivity. In hepatitis mouse models, the antagonistic TNF mutant significantly blocked liver injury caused by inflammation. These results indicate that antagonistic TNF mutants may be clinically useful for anti-TNF therapy and that phage display libraries of protein ligands can be used to select for receptor subtype-selective antagonists.

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*Correspondence should be addressed to:

Haruhiko Kamada, Laboratory of Pharmaceutical Proteomics (LPP) National Institute of Biomedical Innovation (NIBIO) 7-6-8 Saito-Asagi, Ibaraki 567-0085, Osaka, Japan. Phone : +81-72-641-9811, FAX: +81-72-641-9817, e-mail: kamada@nibio.go.jp

Key words tumor necrosis factor- α , phage display system, protein mutant, TNF receptor specific antagonist, anti-TNF therapy

Inflammation is induced by physiological and chemical stimulation and is known to be mediated by the association of many biological factors. Inflammation-mediating proteins, typified by cytokines and chemokines, act in the host defense system by stimulating lymphocytes, macrophages, and endothelial cells to heal external injuries¹. When a productive balance of these mediators collapses, inflammatory exacerbation occurs. Long-term over-expression of cytokines causes autoimmune disease². Thus, development of therapeutic techniques to remedy the imbalance of cytokine production is necessary.

Tumor necrosis factor- α (TNF) is a major inflammatory cytokine and has a central role in host defense and inflammation³. To exert its biological function, TNF binds to two receptor subtypes, TNFR1 and TNFR2, which form homotrimers by preassembling on the cell surface⁴. Deregulation of TNF pro-

duction promotes TNF-dependent pathologies and correlates with the severity and progression of inflammatory diseases such as rheumatoid arthritis (RA)⁵, inflammatory bowel disease⁶, septic shock⁷ and hepatitis⁸. TNF blocking agents (monoclonal antibodies or soluble receptors) have shown significant clinical efficacy in certain inflammatory diseases. The major impact of TNF blocking agents on the immunological system, however, raises some concerns about the safety of this approach, especially with regard to severe infections⁹, malignancies¹⁰ and immune-mediated diseases¹¹. For example, in rheumatoid arthritis and Crohn's disease, studies indicated a higher incidence of tuberculosis reactivation¹² and the induction of demyelination¹³.

Although the distinction between the role of TNFR1 and TNFR2 on the immune system remains unclear, TNF secreted from activated immune cells in these diseases predominantly

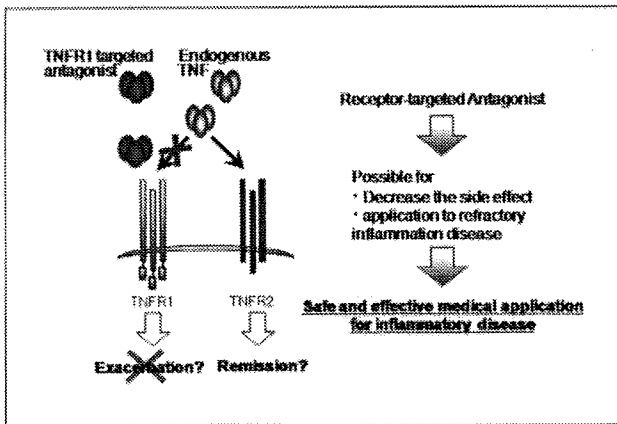


Fig.1 Generation antagonistic protein mutant for receptor targeting

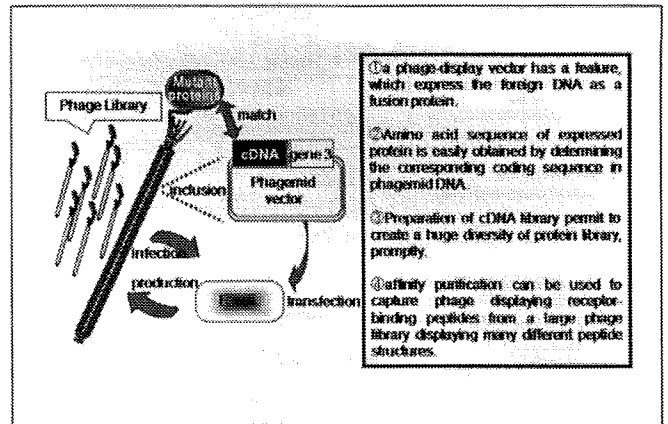


Fig.2 Benefit for engineering protein library using phage display system

activates TNFR1 and accelerates inflammation. In addition, previous studies using animal models of diseases such as arthritis¹⁴ and hepatitis¹⁵ indicated that mainly TNFR1 caused development and exacerbation of inflammation. Moreover, given that in mice lacking the TNFR1 the clinical course of EAE is suppressed both at the pro-inflammatory and the autoimmune phases, the TNFR1 is clearly indicated as an important target for therapy¹⁶. From this perspective, blocking TNFR1 signal transduction may emerge as a powerful and effective therapy for certain inflammatory diseases (Fig.1).

To develop receptor-selective protein ligands, several studies have described useful mutant proteins created by the substitution of amino acids using a site-directed mutagenesis method, as typified by Kunkel's method^{17,18}. It is difficult, however, to obtain an exhaustive and functional panel of protein mutants using this mutagenesis method. Alternatively, the phage display system is a powerful *in vitro* technique that enables polypeptides with desired properties to be selected from a large collection of variants encoded by cDNAs in phagemid vectors (Fig.2). Filamentous phage display of peptide or protein variants has been widely used for rapid selection of protein variants that bind with improved affinity and specificity to target molecules¹⁹. The key feature of such selection schemes is that the genotype of a particular variant packaged inside a virion particle is linked to the phenotype of a displayed protein or peptide that has been fused to phage coat proteins, i.e., the gene III protein. Phage particles can be selected by binding to an affinity matrix propagated in *E. coli* and identified by DNA sequencing. These procedures allow phage libraries to be subjected to a selection step, called "affinity panning". Recovered clones are identified by sequencing and

re-grown for further rounds of selection.

Using the phage display system, we previously isolated a lysine-deficient TNF mutant from a protein library in which all six lysine residues in the TNF molecule, including the receptor-binding site, were simultaneously replaced with other amino acids^{19,20}. This strategy created novel mutant TNFs that exhibited only a slightly different mode of receptor-binding. In the present study, we used the phage display system to isolate novel TNFR1-selective antagonistic TNF mutants that efficiently inhibited a wide variety of TNFR1 mediated effects *in vitro* and *in vivo* without affecting TNFR2-mediated bioactivity.

The selection of amino acids to be altered was based on data from a point mutation study and a TNF structure-function study. Residues (amino acids 89-94) that were shown to contribute to TNFR binding were mapped onto the three-dimensional structure of human TNF. Then, these and other nearby residues were selected for randomization to generate phage libraries (Fig.3). Randomization of each of these residues was performed by PCR with mutated primers in which an NNS codon was incorporated at each randomized position. Each library contained a total of six randomized residues.

To select TNF mutants from phage library that bound strongly to human TNFR1, the mutant TNF phage library was panned against human TNFR1. As a result, we identified ten candidates as TNFR1-selective antagonists and selected the most suitable mutant that possessed the strongest antagonistic activity. To investigate the properties of this antagonistic clone, we examined the binding kinetics and binding specificities of this mutant for TNFR1 and TNFR2 using BIAcore and ELISA techniques, respectively. The antagonistic TNF mutant had an affinity for

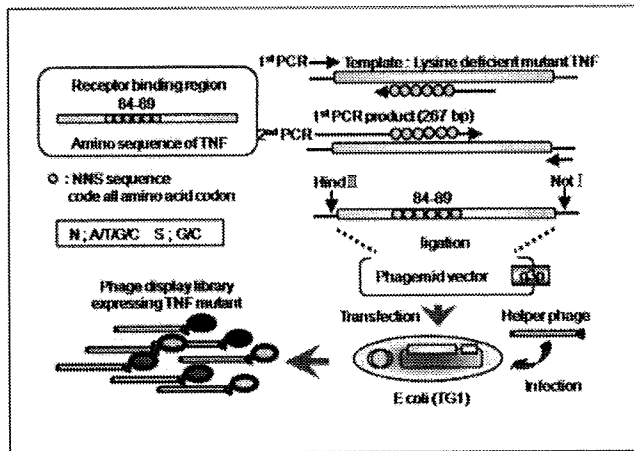


Fig.3 Engineering TNF mutant phage display library

TNFR1 equivalent to wtTNF, but almost no affinity for TNFR2. We also measured the bioactivity of the TNF mutant via TNFR-mediated response assays. The antagonistic TNF mutant bound to TNFR1 but did not transmit the death signal. To determine the ability of the TNF mutant to compete with wtTNF, we measured TNFR1-selective responses in the presence of both wtTNF and TNF mutant. The antagonistic TNF mutant inhibited wtTNF-induced cytotoxicity (Fig.4), caspase activation, and NF- κ B activation through TNFR1 in a dose-dependent manner. These results suggest that the antagonistic TNF mutant is a competitive antagonist, inhibiting TNFR1-mediated pathways.

For the therapy of autoimmune disease, TNF blockades (etanercept, as p75-IgG Fc fusion protein and lenercept as p55-IgG Fc fusion protein) have been developed. However, differences exist in the mechanisms of action of these agents that might confer risks of infection and immunogenicity. There are some reports that tuberculosis disease is a potential adverse reaction from treatment with etanercept. Moreover, antibody formation against lenercept was a significant problem which resulted in significant reduction of the half-life of the receptor. Thus, much is expected from the development of TNF receptor-selective agents that inhibit disease-causing TNF bioactivity without interfering host defense system against infection and antibody formation. In the present report, we generated a receptor-selective antagonistic TNF mutant through the use of phage display. However, there is a possibility of expressing the new function, which binds to another receptor like as TNF receptor superfamily. Therefore, the reasons of showing agonistic or antagonistic activity should be examined via structural analysis of binding sites. We are now analyzing the crystal structures of the complex formed

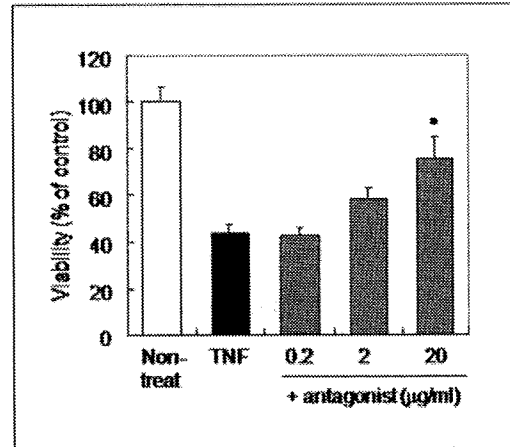


Fig.4 Inhibitory effect of antagonistic mutant TNF on TNF-induced cytotoxicity

Mouse fibrosarcoma L-M cells were treated with wild-type TNF (10 ng/ml), and serial diluted mutant TNF. After 48 hr incubation, ratio of cell death were determined by methylene blue assay.

between the antagonistic TNF mutant and TNFR1 so as to better understand the mechanisms of receptor subtype-selectivity.

While the functions of TNF and its receptors are unclear, their signaling specificities are being examined in many TNF-related studies. In this review, we studied mutant TNF antagonist that bound selectively to TNFR1. The findings from our TNFR1 and TNFR2 study are applicable to the receptors in the TNFR superfamily that do not contain a cytoplasmic death domain. However, we also have produced TNF agonist that binds to TNFR1 and TNFR2. These selective agonists and antagonists are not only therapeutically useful, but also are effective analytical tools for elucidating TNF receptor function. Further functional studies of TNF receptors could uncover interesting receptor biology and may yield additional targets for immunotherapy.

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Research paper

Simple and highly sensitive assay system for TNFR2-mediated soluble- and transmembrane-TNF activity

Yasuhiro Abe^{a,b}, Tomoaki Yoshikawa^{a,c}, Haruhiko Kamada^{a,d}, Hiroko Shibata^{a,e}, Tetsuya Nomura^{a,b}, Kyoko Minowa^{a,f}, Hiroyuki Kayamuro^{a,b}, Kazufumi Katayama^g, Hiroyuki Miyoshi^h, Yohei Mukai^{a,b}, Yasuo Yoshioka^{a,c}, Shinsaku Nakagawa^b, Shin-ichi Tsunoda^{a,d,*}, Yasuo Tsutsumi^{a,b,d}

^a Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

^b Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

^c The Center for Advanced Research and Education in Drug Discovery and Development, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

^d The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

^e Division of Drugs, National Institute of Health Science, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

^f Graduate School of Pharmaceutical Sciences, Kyoto Pharmaceutical University, Misasagi-Nakauchicho 5, Yamashina-ku, Kyoto 607-8414, Japan

^g Allergy & Immunology Project, Tokyo Metropolitan Institute of Medical Science, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan

^h Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan

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ABSTRACT

Drugs that target tumor necrosis factor- α (TNF) are particularly important in the treatment of severe inflammatory progression in rheumatoid arthritis, Crohn's disease and psoriasis. Despite the central role of the TNF/TNF receptor (TNFR) in various disease states, there is a paucity of information concerning TNFR2 signaling. In this study, we have developed a simple and highly sensitive cell-death based assay system for analyzing TNFR2-mediated bioactivity that can be used to screen for TNFR2-selective drugs. Using a lentiviral vector, a chimeric receptor was engineered from the extracellular and transmembrane domain of human TNFR2 and the intracellular domain of mouse Fas and the recombinant protein was then expressed in TNFR1^{-/-} R2^{-/-} mouse preadipocytes. Our results demonstrate that this chimeric receptor is capable of inducing apoptosis by transmembrane- as well as soluble-TNF stimuli. Moreover, we found that our bioassay based on cell death phenotype had an approximately 80-fold higher sensitivity over existing bioassays. We believe our assay system will be an invaluable research tool for studying TNFR2 and for screening TNFR2-targeted drugs.

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1. Introduction

Tumor necrosis factor- α (TNF) is a pleiotropic cytokine that regulates various biological processes such as host defense, inflammation, autoimmunity, apoptosis and tumor cell death through the TNF-receptor 1 (TNFR1) and receptor 2

(TNFR2) (Wajant et al., 2003). TNF/TNFR interaction is considered to be an attractive target for the treatment of refractory diseases, including autoimmune disease and malignant tumors (Aggarwal, 2003; Szlosarek and Balkwill, 2003). In rheumatoid arthritis, for example, biological anti-TNF agents, such as Infliximab and Adalimumab, rapidly reduce signs and symptoms of joint inflammation (Feldmann and Maini, 2003). However, anti-TNF drugs used to treat inflammatory disorders have been reported to increase the risk of infection, in accordance with animal studies (Brown et al., 2002; Nathan et al., 2006).

* Corresponding author. Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Tel.: +81 72 641 9811x2327; fax: +81 72 641 9817.
E-mail address: tsunoda@nibio.go.jp (S. Tsunoda).