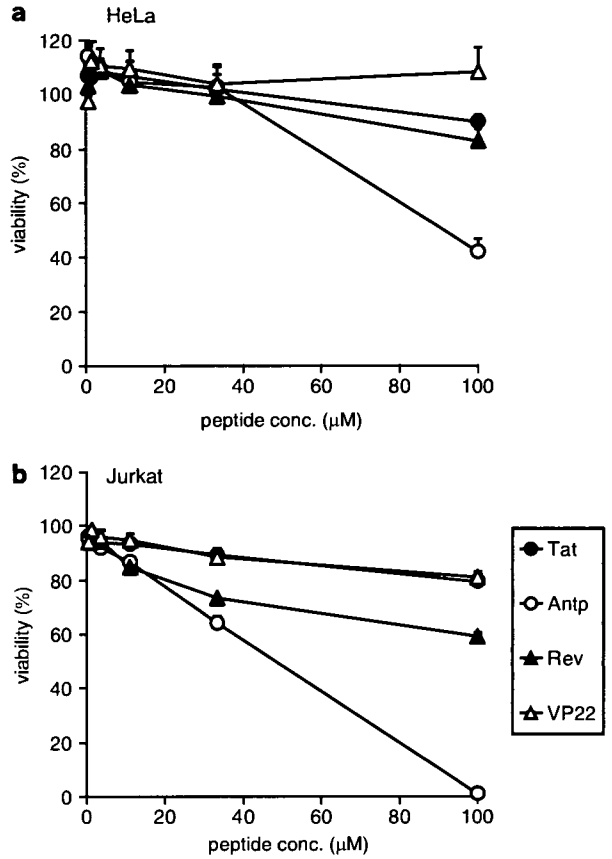

Figure 2 Viability of protein transduction domain (PTD)-treated cells. HeLa cells (**a**) and Jurkat cells (**b**) were incubated with serially diluted biotin-conjugated Tat, antennapedia (Antp), Rev and VP22 at 37 °C. After 24 h, cell viability was analysed using a WST-8 assay. Data shown are the mean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

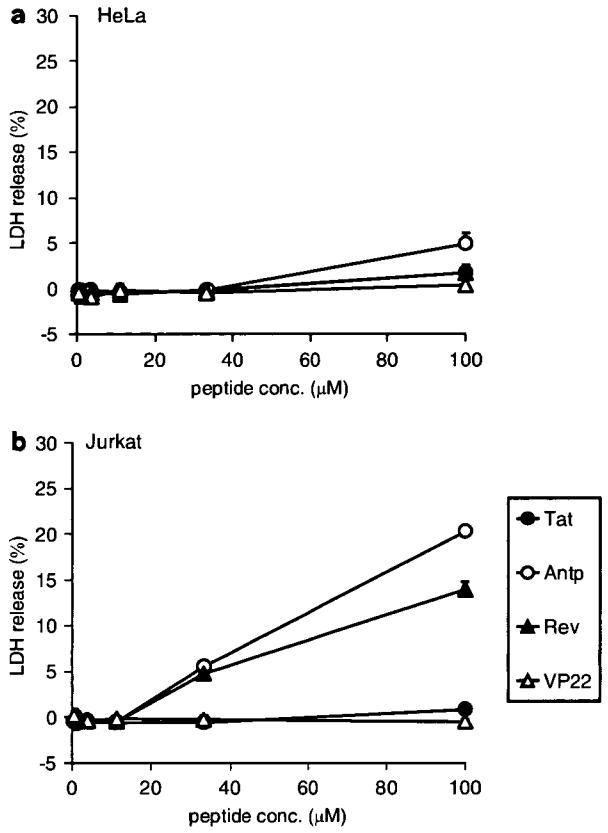

Figure 3 Membrane integrity of protein transduction domain (PTD)-treated cells. HeLa cells (**a**) and Jurkat cells (**b**) were incubated with serially diluted biotin-conjugated Tat, antennapedia (Antp), Rev and VP22 at 37 °C. After 3 h, the release of lactate dehydrogenase (LDH) was analysed. Data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

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 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

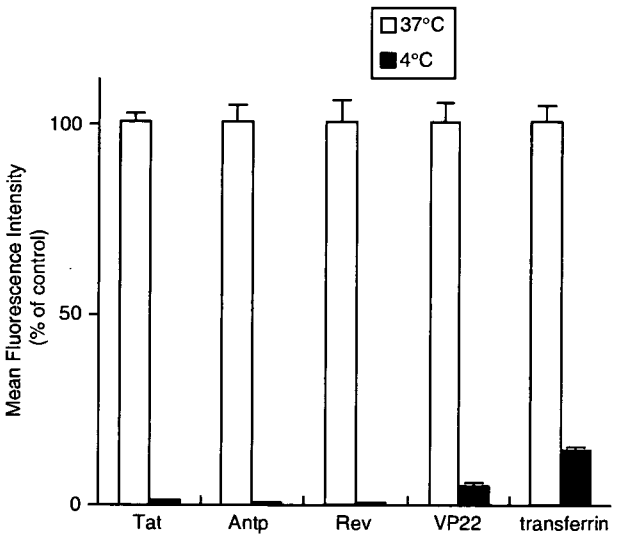

Figure 4 Effects of temperature on protein transduction domain (PTD) transduction efficiency. HeLa cells were preincubated at 37 or 4 °C for 1 h prior to adding FAM-labelled PTDs or fluorescein isothiocyanate-labelled transferrin for 3 h. Cells were washed in trypsin and analysed by flow cytometry. Data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

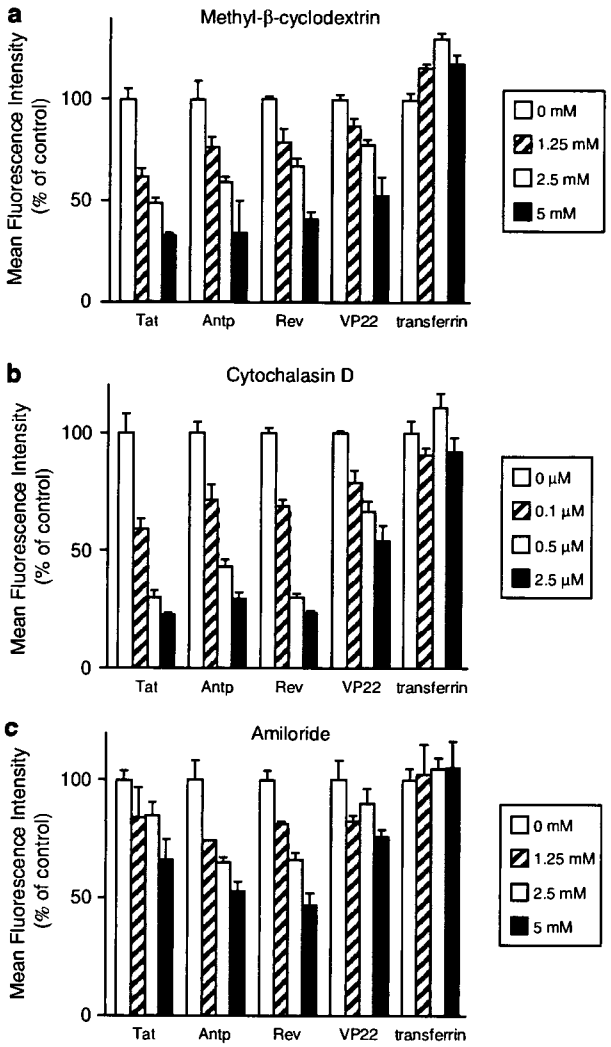

Figure 5 Effects of endocytosis inhibitors on transduction efficiency of protein transduction domains (PTDs). HeLa cells were pretreated with a range of concentrations of (**a**) methyl-β-cyclodextrin, (**b**) cytochalasin D or (**c**) amiloride for 30 min prior to adding FAM-labelled PTDs or fluorescein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

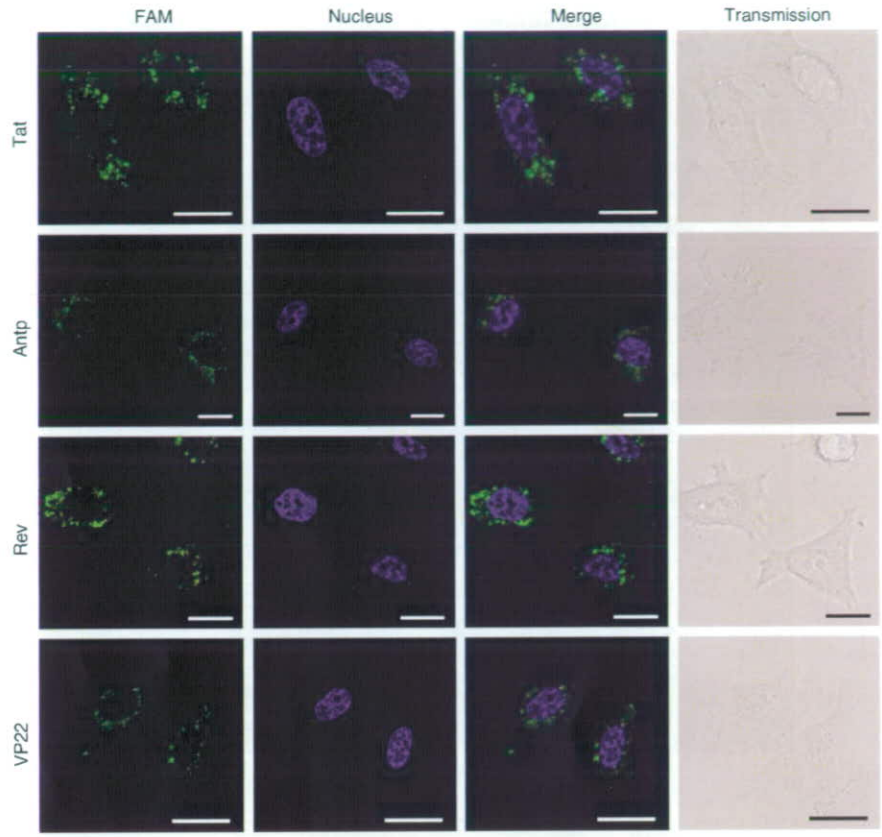

Figure 6 Intracellular behaviour of protein transduction domain (PTD)-FAM in living cells. HeLa cells were treated with 10 μM PTD-FAM for 3 h. Fluorescence images were acquired using confocal laser scanning microscopy and the signals were merged electronically. The nucleus was counterstained with Hoechst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

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,

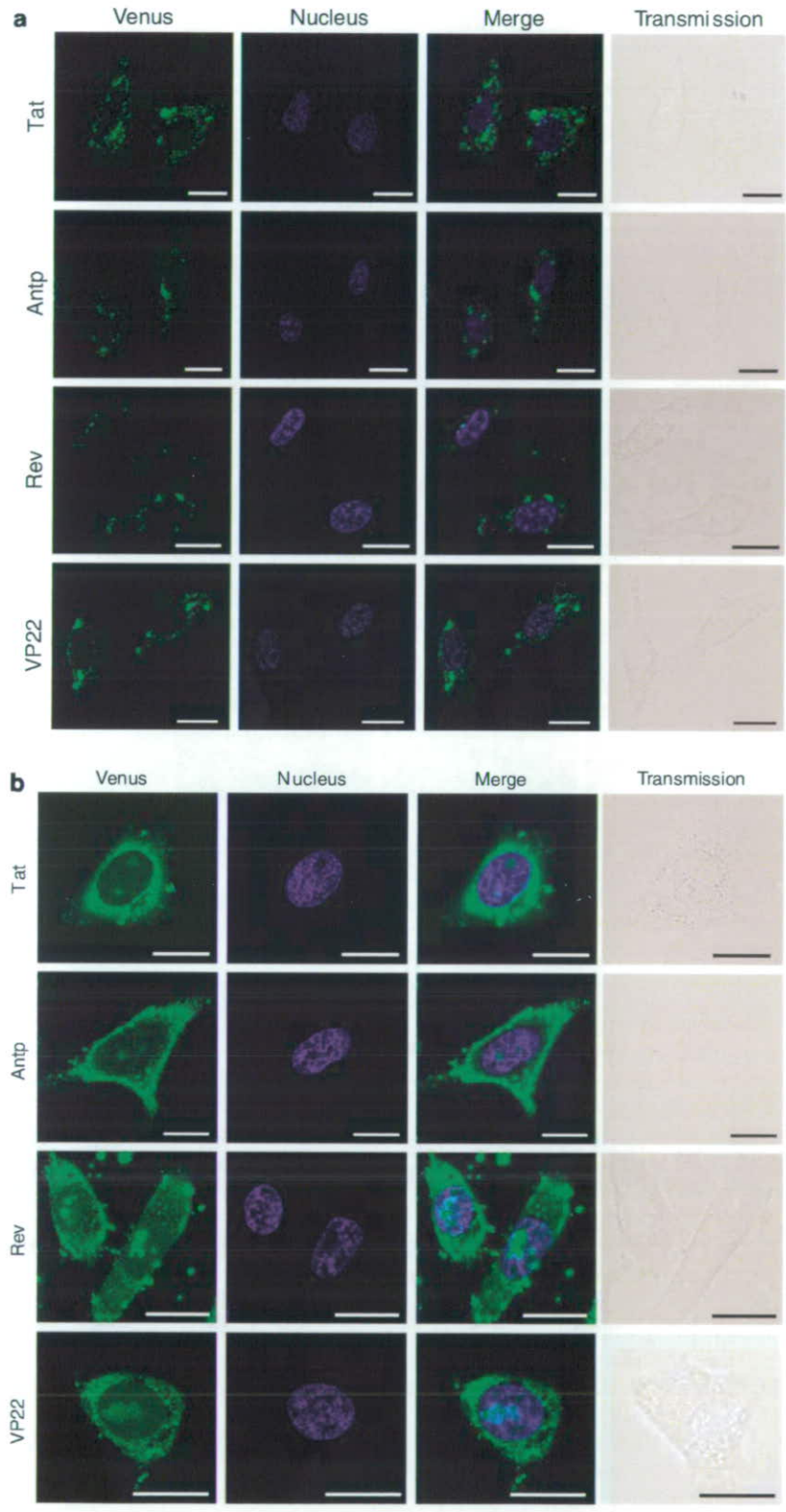

Figure 7 Intracellular behaviour of protein transduction domain (PTD)-Venus in living cells. HeLa cells were treated with 10 μM PTD-Venus alone (**a**) or 10 μM HA2-Tat (**b**) for 3 h. Fluorescence images were acquired using confocal laser scanning microscopy and the signals were merged electronically.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

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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.

Rec.1/25/2007, Acc.4/16/2007, pp512-515

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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 injuries[1]. When a productive balance of these mediators collapses, inflammatory exacerbation occurs. Long-term over-expression of cytokines causes autoimmune disease[2]. 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 inflammation[3]. To exert its biological function, TNF binds to two receptor subtypes, TNFR1 and TNFR2, which form homotrimers by preassembling on the cell surface[4]. Deregulation of TNF production promotes TNF-dependent pathologies and correlates with the severity and progression of inflammatory diseases such as rheumatoid arthritis (RA)[5], inflammatory bowel disease[6], septic shock[7] and hepatitis[8]. 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 infections[9], malignancies[10] and immune-mediated diseases[11]. For example, in rheumatoid arthritis and Crohn's disease, studies indicated a higher incidence of tuberculosis reactivation[12] and the induction of demyelination[13].

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

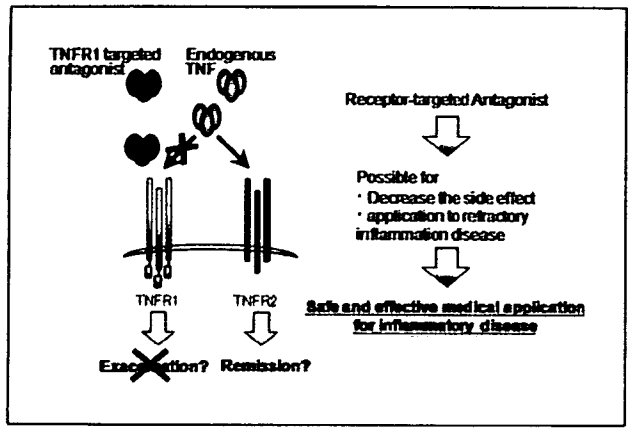

Fig.1 Generation antagonistic protein mutant for receptor targeting

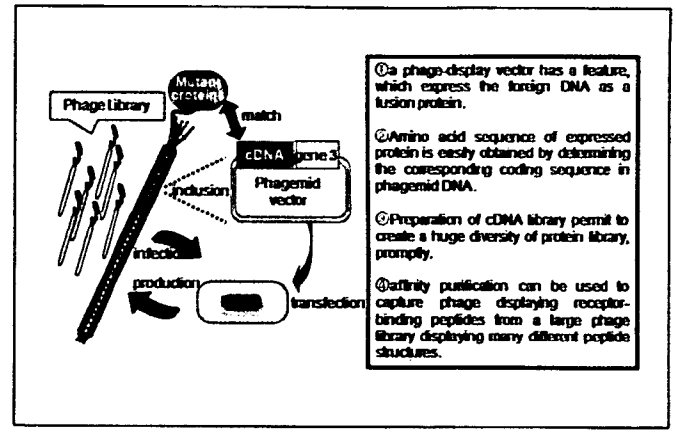

Fig.2 Benefit for engineering protein library using phage display system

activates TNFR1 and accelerates inflammation. In addition, previous studies using animal models of diseases such as arthritis[14] and hepatitis[15] 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 therapy[16]. 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 molecules[19]. 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

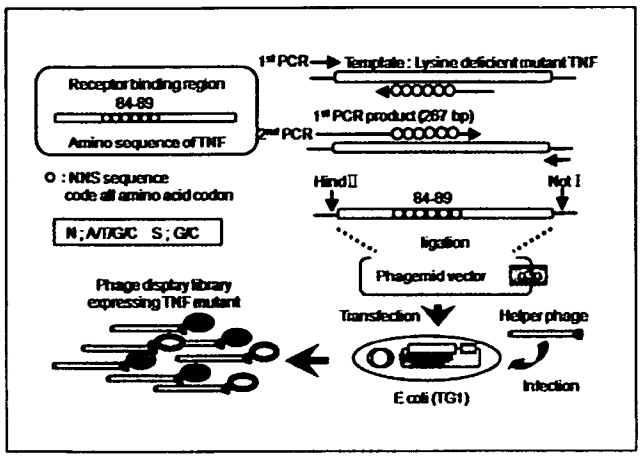

Fig.3 Engineering TNF mutant phage display library

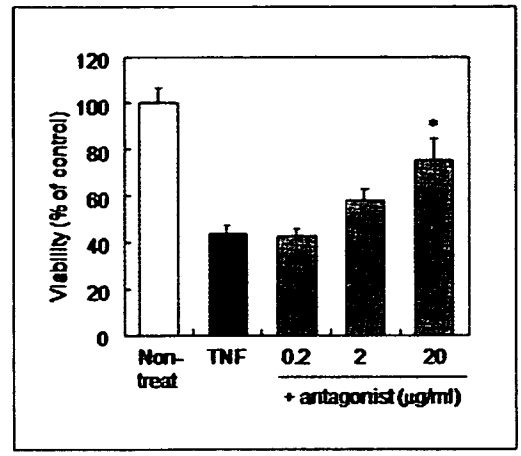

Fig.4 Inhibitory effect of antagonistic mutant TNF on TNF-induced cytotoxicity

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.

TNFR1 equivalent to wtTNF, but almost no affinity for TNFR2. We also measured the bioactivity of the TNF mutant via TNFR-mediating 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-κB 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 (etarnercept, 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 immunogenisity. 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 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.

Acknowledgments

We thank Dr. Shinsaku Nakagawa and Dr. Rie Igarashi for publishing our work in this journal. This study was supported in part by Grants-in-Aid for Scientific Research (No.17689008, 17016084, 17790135, 18015055, 18659047) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, in part by Health and Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan, in part by Health Sciences Research Grants for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, in part by Takeda Science Foundation.

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