Additionally, using FLAG tag selection which removed phage clones causing frame shifts and expressing nonspecific peptides, we successfully ameliorated the method of gene fragment library to be superior in quality and diversity (Fig. 2). However, gene fragments from domain 2 and 3 tended to be deflected to the 5′ end of template. We think this problem will be resolved by appropriately changing a temperature of annealing in RT-PCR for each template. In consideration of this point, we are now constructing gene fragment library of some virus envelope proteins for searching functional peptides.

To assess the availability of our strategy, we tried epitope mapping of anti-TNF-α antibody from TNF-α fragment library. After selection of anti-TNF-α antibody, all amino acid sequences of peptides which strongly bound to the antibody contained amino acids 15–33 sequence of TNF-α (Fig. 5). There are very few reports to confirm that selected phage clones almost encode convergent sequence like this. We predicted there are two reasons: rabbit anti-human TNF-α antibody is easy to recognize the epitope containing an amino acid sequence that differs between human and rabbit TNF-α; and TNF-α fragment library constructed in this study is of dramatically higher quality and diversity than conventional phage libraries. In fact, residues 20–32 of TNF-α have low homology among species and the peptides obtained after the panning contained residues 20, 22, 30, and 31, residues which differ between human and rabbit TNF-α [20]; thus, the peptide was recognized as an epitopic region. Additionally, compared to random peptide library, phage clones bound to the antibody were quite efficiently concentrated from our TNF-α fragment library (Fig. 3). Our system provided a useful strategy for comprehensively searching and identifying functional peptides from various proteins, such as cytokines, extracellular matrix, and coat proteins of viruses. This novel method is likely to be useful for the development of pharmaceuticals, targeting peptides, molecular biological tools, and vaccines.

Acknowledgments

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Improved cytosolic translocation and tumor-killing activity of Tat-shepherdin conjugates mediated by co-treatment with Tat-fused endosome-disruptive HA2 peptide

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Abstract

Tat peptides are useful carriers for delivering biologic molecules into the cell for both functional analysis of intracellular disease-related proteins and treatment of refractory diseases. Most internalized Tat-fused cargos (Tat-cargos) are trapped within the endosome, however, which limits the biologic function of the cargo. In this study, we demonstrated that Tat-fused HA2 peptide ($HA2^{Tat}$), an endosome disrupted peptide, enhanced the endosome-escape efficiency of Tat-cargos. In cells treated with a mixture of fluorescein isothiocyanate-labeled Tat and $HA2^{Tat}$, widespread fluorescence was observed throughout the cytosol. In addition, this $HA2^{Tat}$-mediated cytosolic delivery technique led to enhanced cytotoxicity of Tat-fused anti-cancer peptides, specifically shepherdin. Thus, we improved the function of the delivered molecules by co-treating with $HA2^{Tat}$ and propose that this is a useful method for the delivery of therapeutic macromolecules into the cytosol.

Keywords: Tat; HA2; Protein transduction domain; PTD; Shepherdin; Peptidomimetic antagonist; Peptide blocker

Progress in genomics and proteomics research has led to an increased need for functional annotation of proteomes to allow for the rational choice of particular therapeutic targets from a growing set of candidates. At the same time, the emergence of the interactome of intracellular proteins, such as signal transduction- and protein transport-related proteins, will continue to generate tremendous candidate interactions whose functions need to be clarified and validated in relation with disease [1]. Although small interference RNA- or antisense oligonucleotide-mediated gene knockdown technology are invaluable as primary tools for validation analysis [2,3], these techniques are not always useful for true functional proteomics, because they are not suitable for analysis of post-transcriptional modification, such as phosphorylation, transport to organelles, and protein degradation, and the findings do not always correlate with transcript levels. Therefore, alternative technologies are needed to clarify the function of intracellular candidates, not at the transcript level but at the protein level. Progress in this field will lead to the development of various therapeutic agents.

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Specific inhibition of protein–protein interactions by peptide-blockers, i.e., protein fragments, is a powerful methodology for investigating target validation at the protein level [4–6]. Furthermore, these peptide-blockers possess the potential to be used directly as therapeutic agents. Because many validation targets exist in the cell, it is important that peptide-blockers be delivered directly into cells. There are several recent reports of potent delivery vehicles, known as protein transduction domains (PTD), that can deliver bulky molecular cargos, such as peptides, proteins, oligonucleotides, and nano-particles, into a wide variety of cell types [7–9]. The best-known example of a PTD is the 11 amino acid sequence (Tat; YGRKKRRQRRR) derived from the human immunodeficiency virus type 1 (HIV-1)-Tat protein, and Tat-mediated delivery of peptide-blockers is thought to be useful for evaluating intracellular candidate proteins and developing peptide-based novel therapeutic drugs [10,11]. Recent studies suggested that Tat-fused cargo import is mediated by endocytotic pathways, such as lipid raft-dependent macropinocytosis [12,13]. After internalization via the macropinocytotic pathway, cargos are carried to macropinosomes. For molecules delivered by Tat to function in the cell as both validation probes and therapeutic drugs, they must reach the cytosol. Therefore, macropinosomal escape techniques are needed for the Tat-mediated intracellular delivery of peptide-blockers.

In this context, in the present study we investigated whether the effect of co-treatment with Tat-fused endosome-disruptive peptide ($HA2^{Tat}$) and Tat-fused anti-cancer peptide-blocker shepherdin (shepherdin^{Tat}) induced delivery into the cytosol of tumor cells and enhanced the anti-cancer effect of shepherdin. Although cancer cell treatment with shepherdin peptides, which interfere with the binding between heat shock protein 90 (Hsp90) and survivin, promote the degradation of survivin and increase the sensitivity to apoptosis [14], it is still possible that most of the treated peptides are entrapped in the macropinosomes, thereby limiting the function of shepherdin. With this in mind, we aimed to enhance the cytosolic delivery of peptide-blockers using the N-terminal 20 amino acid peptide of the influenza virus hemagglutinin protein (HA2). HA2 is well-characterized as a pH-sensitive membrane-disruptive peptide that destabilizes lipid membranes at low pH [15,16], and a recent study showed that Tat-fused with HA2 ($HA2^{Tat}$) markedly enhances the disruption of macropinosomes [12]. Therefore, we hypothesized that co-treatment with $HA2^{Tat}$ and Tat-fused peptide-blocker shepherdin^{Tat} will be a promising approach for validating intracellular targets from proteomics analysis as well as the development of effective peptide-based anti-cancer drugs. Here, we evaluated the utility of this strategy using shepherdin peptides.

Materials and methods

Cell lines. HeLa cells, human cervical carcinoma cells, and A549 cells, human lung non-small-cell carcinoma cells, were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells were cultured in α-minimal essential medium (MEMα; Wako Pure Chemical, Osaka, Japan) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. A549 cells were maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemical) supplemented with 10% FBS, 1% L-glutamine, and antibiotics. These cells were cultured at 37 °C, 5% CO_2.

Synthetic peptides. All peptides used in the present study were purchased from GL Biochem Ltd. (Hiroshima, Japan) and their purities of 90% or more were confirmed by HPLC analysis and mass spectroscopy. The sequences of these peptides were YGRKKRRQRRRK-FITC for Tat-fused fluorescein isothiocyanate (FITC^{Tat}), GLFEAIEGFIENGWEG MIDGWYGYGRKKRRQRRR for HA2-fused Tat ($HA2^{Tat}$), KHSSG CAFL for shepherdin, and KHSSGCAFLYGRKKRRQRRR for shepherdin-fused Tat (shepherdin^{Tat}). The Tat sequence is underlined.

Intracellular localization analysis. HeLa cells were cultured on chamber coverglass (Nunc International, Naperville, IL) at 3.0×10^4 cells/well in MEMα supplemented with 10% FBS and incubated for 24 h at 37 °C. Peptide internalization was performed as follows. HeLa cells were co-treated with FITC^{Tat} (10 μM) with or without $HA2^{Tat}$ (2 μM) in Opti-MEM I (Invitrogen, CA) containing 100 ng/ml Hoechst 33342 (Invitrogen). After incubation at 37 °C for 6 h, the medium was replaced with fresh medium and the fluorescence was observed by confocal laser scanning microscopy (Leica Microsystems GmbH, Germany) without cell fixation.

Cytotoxicity assay. HeLa or A549 cells were seeded on 96-well tissue culture plates (Nunc) at 1.0×10^4 cells/well. After incubation for 24 h at 37 °C, the cells were co-treated with various concentrations of shepherdin or shepherdin^{Tat} in the presence or absence of 2 μM (for HeLa cells) or 5 μM (for A549 cells) $HA2^{Tat}$. After 6 h incubation, cell viability was measured using a WST-8 assay kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions.

Flow cytometry analysis. HeLa or A549 cells were seeded on 24-well tissue culture plates (Nunc) at 1.0×10^5 cells/well for 24 h at 37 °C. The cells were co-treated with 10 μM FITC^{Tat} in the presence of 2 μM (for HeLa cells) or 5 μM (for A549 cells) $HA2^{Tat}$ diluted in Opti-MEM I for 6 h. Cells were washed three times with 1 mM EDTA in PBS and treated with 0.25% trypsin to remove the FITC^{Tat} adsorbed on the cell surface and to harvest the cells. Fluorescence was analyzed on a FACSCalibur flow cytometer, and data were analyzed using CellQuest software (Becton–Dickinson, San Jose, CA).

Results and discussion

We first analyzed the subcellular localization of FITC^{Tat} by confocal laser scanning microscopy (Fig. 1). In HeLa cells treated with FITC^{Tat} alone, only punctuate fluorescence was observed intracellularly. We previously confirmed that Tat peptides co-localized in live cells to vesicles with FM4-64, which is a general endosome marker (data not shown). Together, this result and the previous observation indicate that Tat-cargo enters the cell by endocytosis, but most of it is entrapped within the endosomal vesicles. As these results demonstrated that Tat-cargo accumulated in the endosomal vesicles, which severely limited its function, a method that enhances the escape of the Tat-cargo from the endosomes into the cytosol is indispensable for the cargo to exert its function.

Recently, a method for the intracellular delivery of Tat-fused biologically active protein using membrane-disruptive HA2 was studied by several researchers. Wadia et al. reported that $HA2^{Tat}$ markedly enhanced the recombination activity of Tat-fused Cre protein [12]. Additionally, Michiue et al. succeeded in enhancing both nuclear transportation and transcription activity of PTD-fused p53,

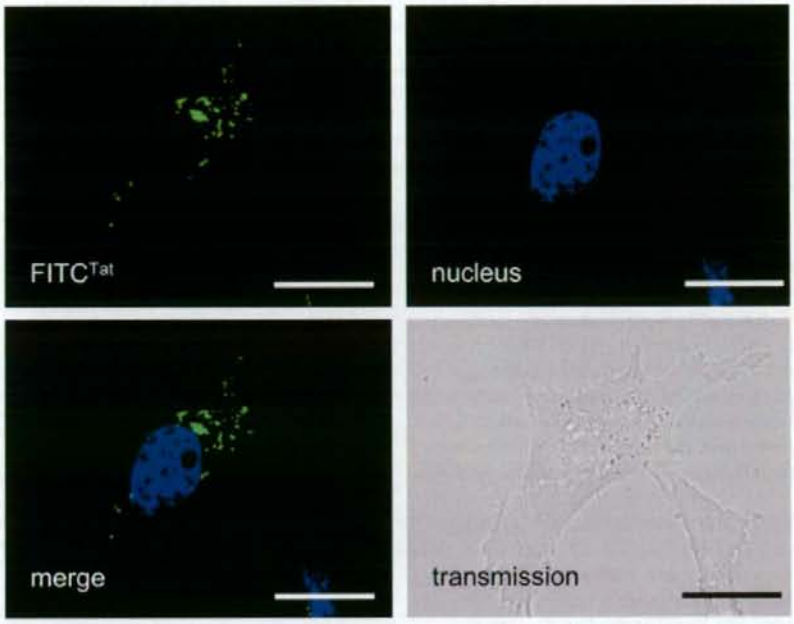

Fig. 1. Intracellular distributions of FITC^{Tat}. HeLa cells were treated with 10 μM FITC^{Tat} alone, and cultured for 6 h. Fluorescence images were acquired by confocal laser scanning microscopy and the signals were digitally merged. The nucleus was counterstained with Hoechst 33342 (blue). Scale bars in each microphotograph indicate 20 μm.

which was directly linked with HA2 [17]. To the best of our knowledge, however, there has been no direct demonstration that HA2 enhances the escape of Tat-cargo from endosomal vesicles. To investigate whether co-treatment with HA2^{Tat} effectively induces the cytosolic translocation of Tat-cargo, HeLa cells were co-treated with HA2^{Tat} and FITC^{Tat} and subcellular localization of FITC^{Tat} was analyzed by confocal laser scanning microscopy (Fig. 2A). The majority of FITC^{Tat} was entrapped in endosomes (Fig. 1). In contrast, co-treatment of HeLa cells with FITC^{Tat} and HA2^{Tat} resulted in dispersed distribution of fluorescence, indicating cytosolic translocation of the Tat-cargo due to disruption of the endosomal vesicles (Fig. 2A). In addition, co-treatment with HA2^{Tat} did not affect the transduction efficiency of FITC^{Tat}, compared with cells treated with FITC^{Tat} alone (Fig. 2B). Thus, we demonstrated that HA2^{Tat} enhances the endosome-escape of Tat-cargos without influencing internalization efficiency.

The model peptide-blocker; survivin-derived shepherdin was developed from a peptidomimetic antagonist of the complex between Hsp90 and survivin [14]. Because knock-down of survivin mRNA induces tumor cell death, survivin is suggested to have a crucial role in tumor development [18,19]. A critical point for the survivin function in tumor cells is its association with Hsp90, which is required to preserve survivin stability [20]. Although transduction of PTD-fused shepherdin (shepherdin^{PTD}) into cancer cells could induce tumor cell death, a high concentration of shepherdin^{PTD} is needed for the induction of growth inhibition in various cancer cells [14]. Therefore, here we used the shepherdin peptide as a model peptide-blocker to investigate the utility of HA2^{Tat}-mediated cytosolic delivery.

The effects of co-treatment with HA2^{Tat} and increasing concentrations of shepherdin^{Tat} on cell viability were investigated using HeLa and A549 cells, which highly express survivin protein [21]. Cells treated with shepherdin alone or co-treated with shepherdin and HA2^{Tat} grew vigorously (Fig. 3). Shepherdin^{Tat} markedly inhibited growth in both HeLa and A549 cells. Co-treatment with HA2^{Tat} and shepherdin^{Tat}, in contrast, resulted in a greater dose-dependent growth inhibition effect than the cells treated with shepherdin^{Tat} alone. The IC_{50} value of co-treatment with HA2^{Tat} and shepherdin^{Tat} (HeLa; 15.61 μM and A549; 32.89 μM) was at least 3-times lower than that of shepherdin^{Tat} alone (HeLa; 48.98 μM and A549; >100 μM; Table 1). These observations indicated that HA2^{Tat} markedly enhanced the cytosolic release of the cargo and the shepherdin-mediated anti-tumor effect. In addition, lung-derived normal human microvascular blood vessel endothelial cells co-treated with HA2^{Tat} and shepherdin^{Tat} showed little growth inhibition (data not shown). Survivin is strongly expressed in embryonic and fetal organs and nearly every human tumor, but has not been reported in differentiated normal tissues [19,22]. Therefore, shepherdin could exert tumor-selective cytotoxic activity. On the other hand, the data presented in Fig. 3 show that HeLa cells are more sensitive to shepher-

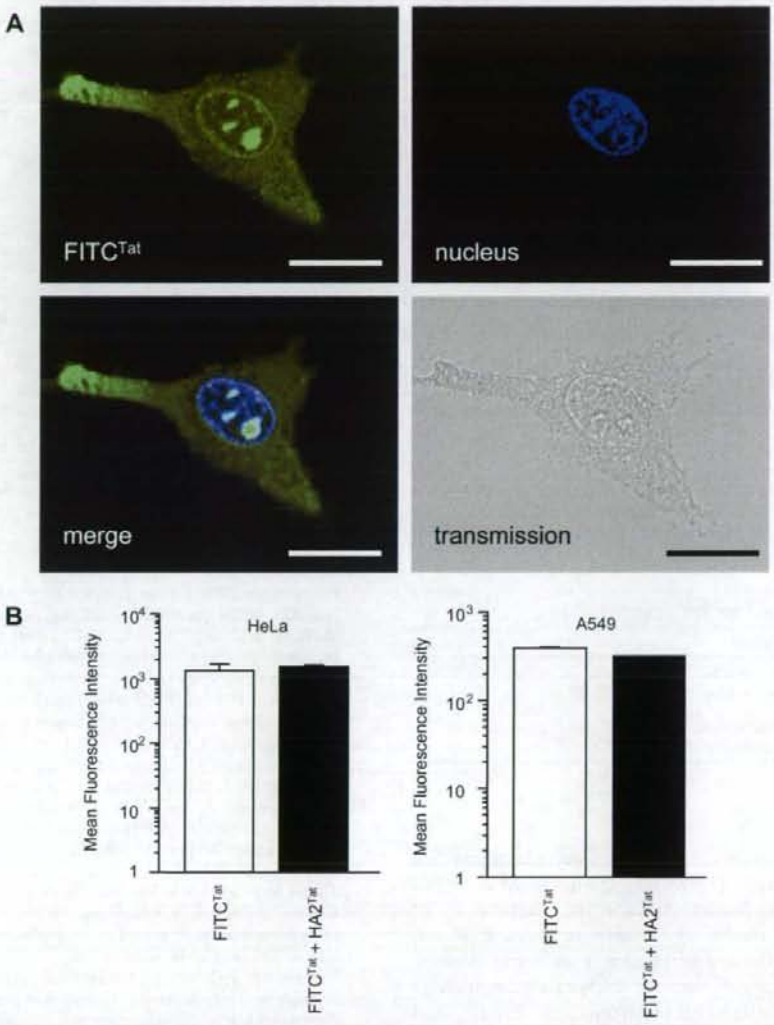

Fig. 2. $HA2^{Tat}$ enhances endosome escape efficiency without influencing the cellular uptake of $FITC^{Tat}$. (A) HeLa cells were treated with 10 μM $FITC^{Tat}$ in the presence of 2 μM $HA2^{Tat}$ for 6 h. The localization of $FITC^{Tat}$ was observed by confocal laser scanning microscopy. The nucleus was counterstained with Hoechst 33342 (blue). Scale bar = 20 μm. (B) HeLa or A549 cells were treated with 10 μM $FITC^{Tat}$ in the presence of 2 μM (for HeLa cells; A) or 5 μM (for A549 cells; B) $HA2^{Tat}$ for 6 h. After trypsin treatment to digest adsorbed-peptides on the cell surface, cells were harvested and analyzed by flow cytometry. Error bars indicate means ± SD of triplicate assays.

din^{Tat} compared with A549 cells. One possibility is the transduction efficiency of $shepherdin^{Tat}$ is different between HeLa and A549 cells. In fact, Fig. 2B shows that the mean fluorescence intensity of $FITC^{Tat}$-treated HeLa cells was relatively higher than that of A549 cells. Nevertheless, almost the same level of growth inhibition was observed in HeLa and A549 cells that were co-treated with $HA2^{Tat}$ and $shepherdin^{Tat}$. Therefore, this $HA2^{Tat}$-mediated cytosolic peptide delivery technique is very useful because it is not affected by cell type.

In the present study, we demonstrated that co-treatment with $HA2^{Tat}$ enhances cytosolic release of Tat-fused peptide-blockers and its biologic activity. Although this co-treatment method is highly useful for enhancing cytosolic peptide delivery, the efficiency of the Tat-mediated intracellular transduction was slightly affected by cell type. Thus, we are currently working to create versatile and highly transducible PTDs using our original PTD-rearrangement method with a phage-displayed random peptide library [23,24].

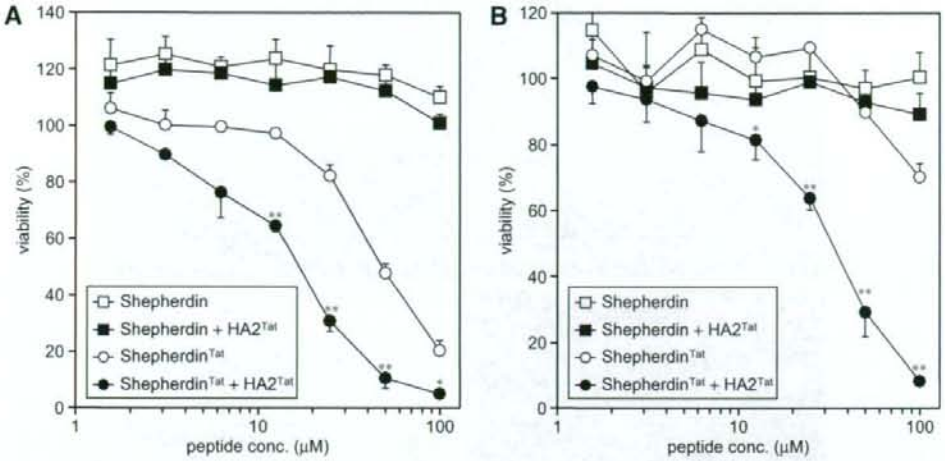

Fig. 3. $HA2^{Tat}$ enhances the cytotoxicity of shepherdin^{Tat}. The cells were treated with shepherdin or shepherdin^{Tat} in the presence of 2 μM (for HeLa cells; A) or 5 μM (for A549 cells; B) $HA2^{Tat}$. After 6 h, the cell viability was analyzed by WST-8 assay. Data are presented as means ± SD of triplicate assays. ** and *, $p < 0.001$ and <0.005, respectively (versus shepherdin^{Tat}).

Table 1
Cytotoxicity of shepherdin^{Tat} in HeLa and A549 Cells

Cell	Peptide	IC_{50} (μM)
HeLa	Shepherdin^{Tat}	48.98
	Shepherdin^{Tat} + $HA2^{Tat}$	15.61
A549	Shepherdin^{Tat}	>100
	Shepherdin^{Tat} + $HA2^{Tat}$	32.89

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Creation of a novel cell penetrating peptide, using a random 18mer peptides library

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Cell penetrating peptides (CPPs) have drawn attention as carriers for intracellular drug delivery. It is commonly believed that TAT peptide is the best carrier among the existing CPPs due to its high translocational activity. Despite considerable research, the cellular uptake mechanism of TAT peptide remains unclear. Additionally, the transduction efficiency of TAT peptide is insufficient for use in intracellular therapy. In this study, we attempted to identify novel CPPs from a random 18mer peptide library using a phage display system. To isolate novel CPPs more effectively, PSIF (protein synthesis inhibition factor) was used with the screening system. Consequently, we isolated 7 novel CPPs from the library and determined by flow cytometry and confocal laser microscopy that these CPPs were taken up into cells. Once the cellular uptake pathway of these CPPs has been determined, it may be possible to use them for intracellular therapy.

1. Introduction

With the progress of proteomics technology over the last few years, many disease-related proteins have been discovered (Kuncl et al. 2002; Lambrechts et al. 2003; St Croix et al. 2000). Many of these proteins reside within the cell. To therapeutically modulate disease-related-proteins, effective methods are needed to deliver other regulatory proteins into cells. Recently, cell penetrating peptides (CPPs) have received considerable attention in this regard (Derossi et al. 1998; Wadia and Dowdy 2002). Examples of CPPs include short peptide segments derived from HIV-1 TAT (Frankel and Pabo 1988; Green and Loewenstein 1988) (13 a.a), *Drosophila* Antennapedia homeodomain proteins (Joliot et al. 1991) (16 a.a), and *Herpes simplex* virus VP22 (Elliott and O'Hare 1997) (17 a.a) are examples of CPP peptides. Because CPPs can translocate various molecules (e.g., peptides, proteins, plasmids, and nucleotides (Astriab-Fisher et al. 2000)) into the cells, CPPs are expected to be useful as carriers for intracellular drug delivery. Of the existing CPPs, TAT peptide is the most effective carrier, and has been used as a carrier to deliver p53 protein for tumor suppression (Li et al. 2002). There are several factors, however, that limit the therapeutic use of CPPs. Firstly, although the mechanism of translocation is thought to be mediated via an endocytic pathway, the precise mechanism of how TAT peptide translocates across the membrane and escapes from the endosome remains unclear. Secondly, the transduction efficiency of TAT peptide is too low to effectively modulate disease-related-proteins. Therefore, for intracellular therapy, novel CPPs are needed that can introduce target proteins into cells more efficiently than existing CPPs by different mechanisms.

Phage libraries expressing polypeptides, such as single-chain antibodies (Imai et al. 2006; Okamoto et al. 2004) or random peptides (Chung et al. 2002; Connor et al. 2001; Scott and Smith 1990), have been used extensively to identify specific molecules with high affinity for target ligands. In the past, novel CPPs have been developed using random peptide phage libraries and cell panning (Hou et al. 2004; Landon and Deutscher 2003; Mi et al. 2003). However, with cell panning, cell-penetrating peptides are difficult to obtain because these peptides bind to the entire cell surface. We previously developed an effective system for screening CPPs using PSIF (protein synthesis inhibition factor). PSIF, a bacteria-derived protein toxin, is non-cytotoxic extracellularly, but once incorporated into cells, it can induce cell death rapidly (Chaudhary et al. 1990; Ogata et al. 1990; Song et al. 2005). CPPs can simply be identified using PSIF-mediated cytotoxicity as an index. In this study, we attempted to create novel CPPs, with greater transduction efficiency cell-penetrating mechanisms that differ from existing CPPs, using a random peptide phage library and a screening system with PSIF.

Table 1: Amino acid sequence of 9 clones selected from random 18 mer peptide library

Clone	Sequence
1	Y A Q Y K I T T A S P G D V K T S N
2	T Y A W Q Y C Q R T G R A L P N T K
3	R K H D A M D S T R R C W P H A P C
4	H N Q R H V K N W P D G F Q R N W S
5	K E Q K N P Q K Q F S S R G P A P N
6	Y P R Y K L Q D T V Q D R L R H R H
7	P K D A Q A S Y T P N N F N L S T T
8	M R Q P K P D T S N Y K D R V K S S
9	M F K G A F T Q Y H S T H E S T E N

2. Investigations, results and discussion

In a first step, a random 18mer peptide phage library was constructed in consideration of cell membrane thickness and the length of existing CPPs. We confirmed that the diversity of the library was 2.0×10^6 CFU, and 9 randomly selected clones consisted of different amino acids as shown by sequence analysis (Table 1). Cell panning was then performed using this library to select clones binding to A431 cells. We evaluated the efficacy of cell panning by calculating the ratio of input to output phage. With successive panning rounds, the ratio of output phage to input phage was increased by approximately 72-fold (Fig. 1). This data suggests that the number of peptide-displaying phages bound to A431 cells was increased. Phagemids of the phage clones selected by cell panning were collected, and the genes encoding peptides were recombined into the PSIF fusion peptide expression vector.

PSIF, which is non-toxic outside the cell, is highly cytotoxic: it inhibits protein synthesis even when only a few molecules are released into the cytosol. PSIF fusion peptides were produced in culture medium of *E. coli*, and applied to A431 cells. We examined the transduction efficacy of peptides into cytoplasm as an index of cytotoxicity of peptide-PSIF fusion protein (Fig. 2). The viability of cells treated with PSIF fusion peptide was calculated by setting

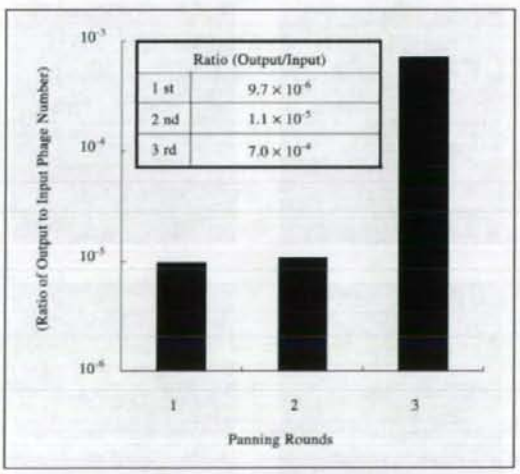

Fig. 1: Selection of binding and internalized phage clones by panning to A431. Phage clones binding to A431 cells with high affinity were collected. The ratio (output phage/input phage) in 3 rounds of panning was calculate. The number of phage clones binding to A431 cells increased with successive rounds of panning

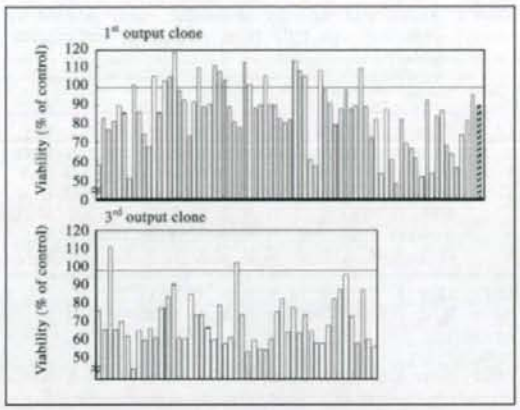

Fig. 2: Cytotoxic activity of randomly selected clones from a random 18mer peptide library. Phage clones were collected after 1 or 3 rounds of panning and genes encoding peptides were recombined with PSIF expression vector. Cytotoxicity mediated by PSIF fusion peptide from randomly selected clones was measured by MTT assay in A431 cells. The viability of A431 cells treated with PSIF fusion TAT peptide was 100%. Clones: (open bar), TAT13: (filled bar)

the viability treated with PSIF fusion TAT peptide at 100%. The rate of clones with viability less than 80% in the first panning output was 16 out of 75 clones (21.3%), and in the third panning it was 28 out of 49 clones (57.1%). We selected 8 clones that introduced PSIF most effectively into the cell, and assessed their cytotoxicity for reproducibility (Fig. 3). The cellular uptake of all PSIF fusion peptides was greater than that of TAT peptide, and their amino acid sequences were analyzed. We then analyzed the phagemid sequences and identified 7 peptides that consisted of different amino acids (Table 2). Existing CPPs consist mainly of basic amino acids and are positively-charged so that they interact with the negatively-charged surface of the cell membrane (Tyagi et al. 2001; Vives et al. 1997; Ziegler and Seelig 2004), and this interaction is important for translocation. Interestingly, 7 peptides were mainly composed of hydrophobic amino acids and contained very few basic amino acids such as lysine and arginine, and were not positively-charged. It has also been reported that cell surface binding of cationic TAT

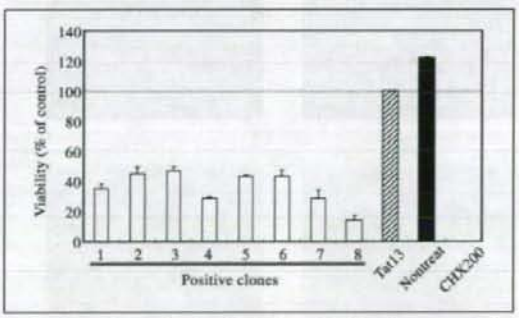

Fig. 3: Cytotoxic activity of positive clones. Positive clones were evaluated with TAT13-PSIF. Eight clones showing strong cytotoxic activity were identified and cytotoxic activity was measured by MTT assay. The viability of A431 cells treated with PSIF fusion TAT peptide was 100%, and the viability of cells treated with 200 mg/ml cycloheximide (CHX200) as a positive control was 0%. Clones: (open bar), TAT13: (hatched bar), Nontreat: (filled bar)

Table 2: Amino acid sequence of positive clones selected by screening with PSIF from random 18 mer peptide library

Clone	Sequence
1	S G E H T N G P S K T S V R W V W D
2	S M T T M E F G H S M I T P Y K I D
3	Q D G G T W H L V A Y C A K S H R Y
4	M S D P N M N P G T L G S S H I L W
5	S P G N Q S T G V I G T P S F S N H
6/7	S S G A N Y F F N A I Y D F L S N F
8	G T S R A N S Y D N L L S E T L T Q
Tat13	G R K K R R Q R R R P P Q
Antennapedia	R Q I K I W F Q N R R M K W K K
VP22	N A K T R R H E R R R K L A I E R

peptide is inhibited by pentosan polysulfate (Rusnati et al. 2001), or heparin (Rusnati et al. 1999). Thus, these peptides might be taken up by different pathways than existing CPPs.

Flow cytometric analysis was performed on the 7 FITC-labeled peptides to evaluate transduction efficacy. The penetration of clones 3 and 6/7 in A431 cells was more than 90% greater than that of FITC-labeled TAT peptide (Fig. 4a). In HeLa cells, clone 6/7 was taken up more effectively than TAT peptides (Fig. 4b). Flow cytometry showed that FITC-labeled clone3 was taken up most effectively in A431 and confocal laser scanning microscopy showed was translocated in A431 cells to the same degree as TAT peptide (Fig. 5). However, results from flow cytometric analysis using FITC-labeled peptides differed from those of the MTT assay using PSIF fusion peptides. With respect to TAT peptide, the 7 peptides fused with PSIF as a cargo molecule were introduced more effectively than peptides fused with low-molecular-weight compound like

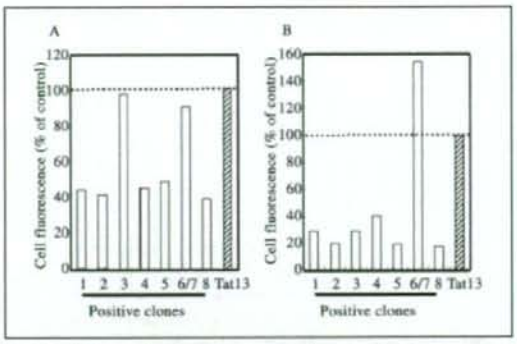

Fig. 4: Cellular uptake of positive clones in the cells. Positive clones were evaluated with FITC-labeled Tat13. A431 cells (A) or HeLa cells (B) were incubated with edium containing FITC-labeled peptide (1 mM) for 3 h. Intracellular translocated peptides were quantified with a a FACScan flow cytometer. Samples were treated with 0.25% trypsin before FACS analysis. The viability of A431 cells treated with PSIF fusion TAT peptide was 100%. Clones: (open bar), TAT13: (hatched bar)

FITC. These data suggest that the molecular weight of the cargo molecule has a considerable effect on the transduction efficiency of CPPs, depending on the characteristics and mechanism of penetration of the CPPs.

In this study, we used a random 18mer peptide library with a PSIF screening system to successfully create novel CPPs that efficiently introduced proteins into cells. We are now investigating the mechanism of penetration of these peptides in studies using inhibitors of cellular uptake pathways. We are also attempting to clarify the relationship between molecular weight of cargo molecules and transduction efficiency with several CPPs. Our data may contribute to the development of intracellular therapy with disease-related proteins.

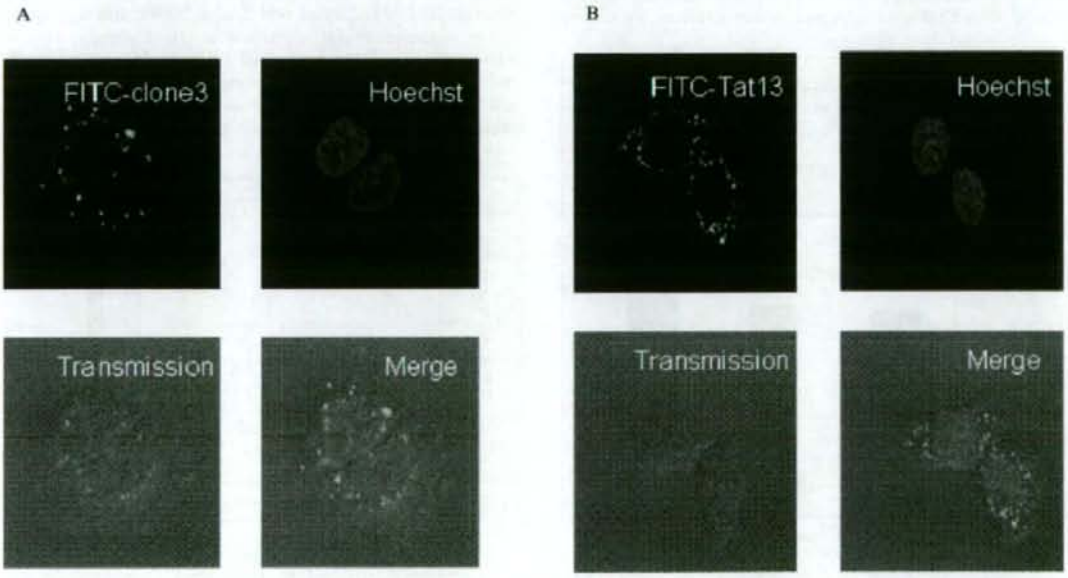

Fig. 5: Intracellular distribution of clone3 in living cells. A431 cells were cultured with FITC-labeled clone3 (A) or TAT peptide (B) for 3 h. Cells were washed and nuclei were stained with Hoechst 33342. Cells were examined by confocal laser microscopy

3. Experimental

3.1. Construction of gene fragment library coding random 18mer amino acids peptide

Gene fragments encoding random 18mer amino acid peptides were produced with Klenow fragment and the following primers: P-oligo1 (5′-GATTACGCCAAGCTTTGGAGCCTTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTATGCGGCCCAGCCGGCCATGGCC-3′), P-oligo4(5′-CGGCGCACCTGCGGCCGCSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNGGCCATGGCCGGCTGGGCCGCATAGAA-3′).

To anneal the primers, 25 pmol each of P-oligo1 and P-oligo4 were mixed with 10 × Klenow buffer and incubated at 96 °C for 10 min, 70 °C for 5 min, and 16 °C for 10 min. Klenow fragment (TOYOBO) and 10 mM dNTP were then added to the reaction mixture and incubation was continued at 37 °C for 1 h. The purified sample was extracted from agarose gel using a QIAquick Gel Extraction Kit (QIAGEN). Gene fragments were then amplified by PCR. PCR reactions were cycled 35 times at 96 °C for 1 min, 65 °C for 1 min, 68 °C for 1 min, using pCANTAB HindIII (5′-GGAAACAGCTATGACCATGATTACGCCAAG-3′), and NotI extension (5′-GTAAATGAATTTTCTGTATGAGG-3′) as primers. The gene library and pCANTAB5E phagemid vector were digested with Hind III and Not I, and ligated with T4 ligase to display random 18mer amino acid peptides on the phage surface as fusion proteins with gene 3 protein. The phage library was prepared as previously described. Sequence analysis of randomly selected clones was performed.

3.2. Cell panning

A431 cells (epidermoid carcinoma, human) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. A431 cells (1.0×10^6 cells/2 ml/well) were seeded in 6-well plates and grown for 24 h. The wells were washed three times with PBS, and then incubated at 37 °C for 2 h with 2 ml/well Opti-MEM®I Reduced-Serum Medium (Invitrogen™ Life Techologies) containing 2% BSA. Medium was removed from each well, and the cells were incubated at 37 °C for 2 h with the purified phage library ready blocked with Opti-MEM containing 2% BSA. After washing the wells with PBS 20 times, the bound phages were eluted by incubating the wells with 100 mM HCl solution at 4 °C for 10 min. Eluted phages were immediately neutralized with 1 M Tris-HCl buffer pH 8.0 and were recovered as output phages. For the next panning, the eluted solution was added to log phase *E. coli* TG1 cells (Stratagene). The ratio of output phage to input phage was calculated to determine the effects of cell panning. Input phage and output phage were diluted in 2YT medium containing 50 µg/ml ampicillin and 2% glucose, and added to log phase *E. coli* TG1 cells. After incubation at 37 °C for 1 h, *E. coli* solution was seeded on LB (50 µg/ml ampicillin and 2% glucose) plates, and the number of colonies formed were counted.

3.3. Recombination to PSIF-expression vector

Protein synthesis inhibitory factor (PSIF, PE fragment) is an approximately 40kD fragment of the bacterial exotoxin (GenBank Accession No. K01397) derived from *Pseudomonas aeruginosa* (ATCC strain No. 29260). PSIF lacks its cell binding domain, and is the truncated form of *Pseudomonas aeruginosa* exotoxin, which is a non-toxic protein outside of the cell. One (Dr. Tsunoda) of us cloned the cDNA for PSIF from *Pseudomonas aeruginosa*, Migula by PCR using the primer set 5′-GAT GAT CGA TCg cgg ccg caG GTG CGC CGG TGC CGT ATC CGG ATC CGC TGG AAC CGC GTG CCG CAg act aca aag acg acg acg aca aaC CCG AGG GCG GCA GCC TGG CCG CGC TGA CC-3′ and 5′-GAT CGA TCG ATC act agt CTA cag ttc gtc ttt CTT CAG GTC CTC GCG CGG CGG TTT GCC GGG-3′. The fusion protein, denoted peptide-PSIF, consisted of peptide at the N-terminus and a PSIF at the C-terminus. First, the peptide gene containing phagemid vectors were recovered with QIAprep® Miniprep Kit (QIAGEN) and digested with HindIII and NotI. The peptide gene fragments encoding random 18mer amino acids were then subcloned into PSIF Expression Vector, which is modified from pCANTAB-5E.

3.4. Cytotoxicity assay by PSIF-fusion peptide

PSIF display phagemid was was transfected into TG1 cells, and individual TG1 clones were selected and grown at 37 °C in 96-well plates. Supernatants of TG1 cells were prepared for MTT assay. After seeding of A431 cells treated with 10 µg/ml cycloheximide at 2.0×10^4 cells/50 µl/well, 35 µL of Opti-MEM and 5 µl of supernatant were added to each well. After incubation at 37 °C for 24 h, 10 µL of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) solution was added. MTT assays were carried out according to the manufacturer's protocol.

3.5. Flow cytometry

A431 and Hela cells (2.0×10^5 cells/well in 12-well plates) were grown for 24 h and incubated with each FITC-labeled clone peptide or TAT peptide at 37 °C for 3 h. The cells were washed twice with PBS and once with PBS/EDTA, and then treated with 0.25% trypsin for 10 min. The cells were collected, and fixed with PBS (500 µl/well) containing 1% PFA. The fluorescence intensities of the samples were measured with a FACS calibur (Becton Dickinson) detector.

3.6. Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed using a Leica TCS SP2 (Leica). A431 cells were seeded at 2.25×10^4 cells/well in 4-well chamber slide glasses. After overnight incubation, cells were washed once with phenol red-free DMEM containing 10% FBC and 1% antibiotics. Each peptide (TAT peptide and clone3) and nuclear stain marker Hoechst33342 (molecular probes) were added at a concentration of 100 ng/ml in 500 µl of serum-free Opti-MEM), and then incubated at 37 °C for 3 h. The cells were subsequently washed three times and then analyzed.

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Creation of Novel Cell-Penetrating Peptides for Intracellular Drug Delivery Using Systematic Phage Display Technology Originated from Tat Transduction Domain

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Many biologically active proteins need to be delivered intracellularly to exert their therapeutic action inside the cytoplasm. Cell penetrating peptides (CPPs) have been developed to efficiently deliver a wide variety of cargo in a fully biological active form into a range of cell types for the treatment of multiple preclinical disease models. To further develop this methodology, we established a systematic approach to identify novel CPPs using phage display technology. Firstly, we screened a phage peptide library for peptides that bound to the cell membrane. Secondly, to assess functionality as intracellular carriers, we recombined cDNAs of binding peptides with protein synthesis inhibitory factor (PSIF) to create fusion proteins. Randomly chosen clones were cultured and expression of peptide-PSIF fusion proteins induced, followed by screening of protein synthesis activity in cells. Using this systematic approach, novel and effective CPPs were rapidly identified. We suggest that these novel cell-penetrating peptides can utilized as drug delivery tools for protein therapy or an analytical tool to study mechanisms of protein transduction into the cytoplasm.

Key words cell penetrating peptide; phage display; Tat

Many biologically active compounds, including a variety of large molecules, need to be delivered intracellularly to exert their therapeutic action inside the cytoplasm or within the nucleus or other specific organelles. An important requirement in the use of proteins in this context (ex. kinases, phosphatases, transcriptional factors) is the ability of these molecules to efficiently penetrate across the cell membrane. However, the plasma membrane of cells is largely impermeable to proteins and peptides. Recently, it was discovered that certain short peptide sequences, composed mostly of basic, positively charged amino acids (*e.g.* Arg, Lys and His), have the ability not only to transport themselves across cell membranes,[1–3] but also to carry attached molecules (proteins, DNA, or even large metallic beads) into cells.[4–6] These basic sequences are now commonly known as protein transduction domains or cell-penetrating peptides (CPPs) and have been successfully employed to transport cargo proteins across a variety of cell membranes.[7] Cellular delivery using CPPs has several advantages over conventional techniques; indeed, it is efficient across a range of cell types and can be applied to cells *en masse*.[8]

It has been proposed that the Tat transduction domain of HIV is first endocytosed into a caveola compartment and secondarily released into the cytoplasm, following vesicle disruption.[9] Once CPP binds to the cell surface heparan sulfate proteoglycan (HSPG), the CPP-fused protein is internalized *via* a lipid raft-mediated pathway. Additionally, the mechanisms responsible for CPP mediated cargo internalization estimated with regard to enter the cells *via* macropinocytosis[10] and/or through clathrin-mediated endocytosis,[11] or possibly *via* an unknown alternative mechanism. In spite of some common features of these peptides, particularly their highly cationic nature, their structural diversity has fuelled the idea that the penetrating mechanism is not the same for CPPs of different types. As such, the mechanism(s) of internalization of CPPs has not been resolved yet.[7]

Given the potency of the Tat-derived CPPs in mediating the cellular uptake of small and large macromolecular cargos, as demonstrated within the last few years, a large number of laboratories have exploited this system as a tool for transcellular penetration of cultured cells.[12] Most of these applications are based on the fusion of the protein transduction domain of Tat to the protein of interest, either at the N- or C-terminus, followed by addition of the recombinant fusion protein to the culture medium of the cells of interest. It is clear that CPPs are novel vehicles for the rapid translocation of cargo into cells, and exhibit the properties that make them potential drug delivery agents.[13] However, there are problems in respect to a decrease in the rate and efficiency of translocation for large proteins that has not yet been overcome. Accordingly, a large number of different CPPs have been explored to promote translocation of various types of useful cargo, ranging from small molecules to proteins and large supramolecular particles, with great efficiency and reasonable velocity.

We previously showed that the gene III proteins (pIII) of M13 filamentous phage could be used to display mutant protein, with these modified proteins showing fully functional binding to receptor and consequent biological activities.[14,15] Recently, we established a novel whole cell panning method,

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which selected cell adhesive phage-displayed peptides and, subsequently, a cohort of these peptides having cell penetrating qualities *via* the use of PSIF (protein synthesis inhibitory factor). In this study, we constructed a Tat-based mutant peptide library using this phage display system. Moreover, we demonstrated the direct selection of a unique cell-binding activity utilizing whole cell panning methods and the screening of internalizing peptide using peptide-PSIF fusion protein.

MATERIALS AND METHODS

Cell Line Human epidermoid carcinoma A431 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5.0% CO_2 at 37 °C. Human adenocarcinoma Hela cells were grown in DMEM supplemented with NEAA and 10% FCS. Chinese hamster ovary (CHO)-K1 cells were grown in Ham's F12K medium supplemented with 2 mM L-glutamine and 10% FCS.

Preparation of Phage Peptide Library Primers shown below and used in library construction were purchased from Hokkaido System Science Inc. The phage-display vector pCANTAB-5E was used as a phagemid vector for the generation of the peptide-pIII fusion gene repertoires (Fig. 1). To construct a DNA fragment library encoding 13 amino acid peptides, primers P-oligo1 and P-ologo2 were annealed and elongated with the Klenow fragment in the presence of nucleotide triphosphates. These cDNA-encoding peptide library products were purified with QIAquick® Gel Extraction Kit (QIAGEN) and used as templates for PCR with primers pCANTAB-Hind and Not I Ext to generate the pIII fusion gene repertoires. The peptide-encoding genes were digested with the restriction enzymes HindIII and NotI, agarose gel-purified, and ligated into pCANTAB-5E, which was cut with the same restriction enzyme. The ligated products were electroporated into *E. coli* TG1 cells, plated on modified LB medium (Invitrogen) containing 2% glucose and 50 μg/ml ampicillin, and then incubated overnight at 37 °C. The clones were scraped off the plates into 2YT medium with 10% glycerol and subsequently stored at −70 °C. P-oligo1; 5′-GAT TAC GCC AAG CTT TGG AGC CTT TTT TTT GGA GAT TTT CAA CGT GAA AAA ATT ATT ATT CGC AAT TCC TTT AGT TGT TCC TTT CTA TGC GGC CCA GCC GGC CAT GGC C-3′, P-oligo2; 5′-CGG CGC ACC TGC GGC CGC SNN SNN CGG SNN SNN SNN CTG SNN SNN SNN SNN SNN ACC GGC CAT GGC CGG CTG GGC CGC ATA GAA AGG-3′, pCANTAB-Hind; 5′-GGA AAC AGC TAT GAC CAT GAT TAC GCC AAG-3′, Not I Ext; 5′-GCG GCC TTG TCA TCG TCA TCC TTG TAG TCT GCG GCC GC-3′.

Rescue of Peptide-Phage To rescue the peptide-phage library, 11 of 2YT medium, containing 2% glucose, and 100 μg/ml of ampicillin, was inoculated from the glycerol stock library. The culture was shaken at 37 °C until OD600 nm=0.4 and 3.2×10^8 plaque forming units of M13KO7 helper phage (Invitrogen) were added. After 30 min incubation at room temperature with shaking, the culture was centrifuged and the pellet recovered. The pellet was then incubated with 50 μg/ml of kanamycin and 100 μg/ml of ampicillin within 2YT medium and grown for 6 h at 37 °C. The phage was purified by standard polyethylene glycol precipitation and filtration with a 0.45 μm PVDF filter (Millipore). Peptide-phage which did not express the objective peptide were removed by a FLAG panning method, as described previously.

Biopanning Method We used a slightly modified procedure from that found in the literature. Briefly, 1.0×10^6 A431 cells were harvested in 6 well culture plates and incubated for 24 h at 37 °C within a 5.0% CO_2 incubator until the logarithmic phase of growth was reached. The culture plates were washed with PBS 3 times and 2% BSA Opti-MEM® (Invitrogen) added 2 h prior to the addition of the peptide-phage. Cells were incubated with the peptide-phage library for 2 h at 37 °C with shaking every 15 min during the round of panning. Following this, the cells were washed twenty times with PBS at room temperature. After washing, the cells were lysed with 1 ml of 100 mM HCl and neutralized with 0.5 ml of 1 M Tris–HCl, pH 8.0. One-hundred microliters lysate was used

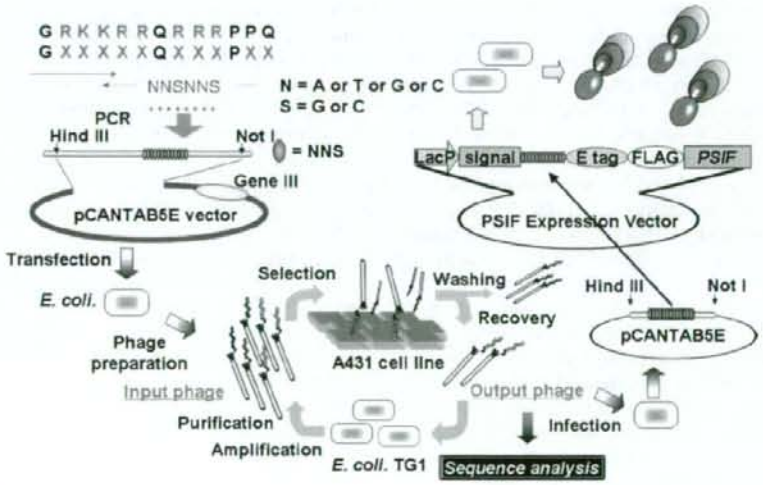

Fig. 1. Schema for Screening CPPs

to infect 0.3 ml of *E. coli* TG1 cells, with phage being rescued as described above and used in the next round of selection.

Expression and Purification of a Peptide-PSIF Fusion Protein Protein synthesis inhibitory factor (PSIF, PE fragment) is an approximately 40 kD fragment of the bacterial exotoxin (GenBank Accession No. K01397) derived from *Pseudomonas aeruginosa* (ATCC strain No. 29260). PSIF lacks its cell binding domain, and is the truncated form of *Pseudomonas aeruginosa* exotoxin, which is a non-toxic protein outside of the cell. One (Dr. Tsunoda) of us cloned the cDNA for PSIF from *Pseudomonas aeruginosa*, Migula by PCR using the primer set 5′-GAT GAT CGA TCg cgg ccg caG GTG CGC CGG TGC CGT ATC CGG ATC CGC TGG AAC CGC GTG CCG CAg act aca aag acg acg acg aca aaC CCG AGG GCG GCA GCC TGG CCG CGC TGA CC-3′ and 5′-GAT CGA TCG ATC act agt CTA cag ttc gtc ttt CTT CAG GTC CTC GCG CGG CGG TTT GCC GGG-3′. The fusion protein, denoted peptide-PSIF, consisted of peptide at the N-terminus and a PSIF at the C-terminus. First, the peptide gene containing phagemid vectors were recovered with QIAprep® Miniprep Kit (QIAGEN) and digested with HindIII and NotI. The peptide gene fragments were then subcloned into PSIF Expression Vector, which is modified from pCANTAB-5E. The fusion proteins were expressed and collected within the supernatant from *E. coli* TG1 cells, with the supernatant being used for cell viability assays.

Cell Viability A431 cells (2.0×10^4) were incubated with 35 μl Opti-MEM® and 10 μl cycloheximide (100 μg/ml) in 96 well plates. Cells were treated with 5 μl peptide-containing supernatant for 24 h and the cell viability was monitored by MTT assay. Twenty-four hours after addition of the peptides, 10 μl of 5 mg/ml MTT (Dojindo) were added to each well and the cells were further incubated at 37 °C for 4 h. Subsequently, the insoluble formazan crystals were solubilized in a solution of 20% SDS containing 0.01 N HCl. Absorbance measurements were taken at $\lambda=595$ nm with background subtracted at $\lambda=655$ nm. Each sample point was performed in duplicate.

FACS Analysis The specific cell binding activities of peptides towards A431, Hela, CHO-K1 cells were measured by FACScan (Becton Dickinson). Cells were grown in tissue culture flasks to late logarithmic phase. Culture medium was renewed 2 h prior to the addition of the peptide-phage. FITC-labeled peptides were purchased from Genenet Co., Ltd. and 1×10^5 cells were incubated with FITC-labeled peptide for 3 h at 37 °C. For the endocytosis inhibitor assays, FACS analysis was performed after pre-treating A431 cell monolayers at 37 °C with 10 mM methyl-β-cyclodextrin (MβCD; caveola-mediated endocytosis inhibitor) or amiloride (macropinocytosis inhibitor) in serum-free Minimal Essential Medium (MEM) for 30 min, followed by a 1-h co-incubation with FITC labeled-peptide. After three washes with PBS, 0.25% trypsin solution (Gibco BRL) was added and incubated for 15 min to digest non-specific binding peptides. After three additional washes, cells were resuspended in PBS/4% paraformaldehyde and analyzed using FACScan.

RESULTS

Construction of Phage Peptide Library and Quality Check The pCANTAB-5E phagemid library used here has previously been screened successfully for mutant protein which binds to receptors.[14,15] Additionally, we previously reported the identification and characterization of a series of cationic peptides, similar to the CPP derived from Tat, which are able to penetrate large protein complexes into a wide variety of cells, including fibroblasts. Here, we made a novel phage peptide library, which altered ten amino acids within the Tat transduction domain (13 amino acids). The library of the TAT-based CPPs was made *via* the annealing and elongation of two mutated primers, followed by PCR amplification and cloning into a phage expression system The peptide-encoding cDNA library was placed into a phagemid vector and expressed as a fusion protein with phage coat protein, pIII. We confirmed the identity and sequence distribution of this phage peptide library by DNA sequencing (Table 1). In this context, eight clones which were sequenced showed independent sequences, highlighting the distribution of this phage peptide library.

Concentration of Binding Peptides with a Cell Panning Method The constructed peptide phage library was selected *in vitro* against A431 cells. Selection was performed as described in the Materials and Methods section, with a view to enriching for peptides displaying cell binding activity. With respect to the phage panning and amplification processes, which were repeated for one to four rounds, the output/input ratio was found to increase in a manner dependent on cycle number (Fig. 2). These results indicated that the peptides having an affinity for A431 cells were enriched gradually by this cell panning approach.

Identification of A431 Cell Binding Peptides Peptide clones that became internalized in A431 cells were isolated by four rounds of selection. In order to select only internalized phage-derived peptides, cells were incubated with super-

Table 1. Random TAT Peptide Library Sequence before Panning

Clone	Sequence
1	GMHINGQSNPPHA
2	GGMHESQSHMPGD
3	GTQAFLQQFEPWI
4	GIKHSPQQISPRW
5	GILCIQQDHQPLG
6	GFKLSSQAVAPLQ
7	GSIRAPQGDSPWP
8	GTRHGIQTQPPNN

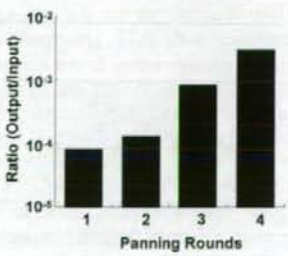

Fig. 2. Enrichment of Phage Clones by Biopanning

A431 cells were incubated with 4×10^{10} titer phage. After washing with PBS, binding phages were recovered and the titer was determined. The index of enrichment was evaluated with input/output ratio.

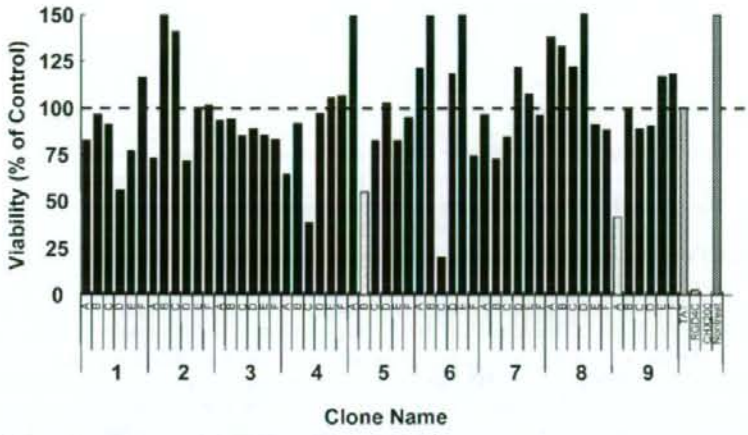

Fig. 3. Measurement of Penetrating Activity as an Index of Cytotoxicity to A431 Cells

PSIF-CPP fusion genes were transformed into TG1 cells, with resulting fusion proteins then recovered from the supernatant. The cytotoxicity of the supernatant was then assessed to examine the activity of the CPPs. Cell viability in response to fusion proteins was compared with that exhibited by exposure to the parent Tat13 peptide (cell viability=100%), and by exposure to 200 μg/ml cycloheximide (CHX; cell viability=0%).

natant collected from *E. coli* cells. After four rounds of selection, an enrichment in the order of 10^5 was obtained. The random insert region of the single-stranded DNA from individual clones, following the fourth round of selection, was sequenced and the amino acid coding sequence deduced. To identify peptides capable of facilitating internalization, we developed a screening method using an M13 peptide phage display library. We incubated 1×10^6 A431 cells, with 4×10^{10} phage from a 13-mer peptide M13 phage display library for 2 h at 37 °C. Following four rounds of screening, we isolated phage from 28 plaques and determined the identity of the encoded peptides by DNA sequencing (Table 2). Twenty-eight of the peptide sequences were found in at least 15 independent plaques and were selected for further analysis.

Evaluation of Cell Penetrating Activity of CPPs Following creation of the peptide library (input phage library), we expressed these Tat-based CPPs as fusion proteins with PSIF. From this, 54 candidates were found that exhibited lower cytotoxic activity than the parent Tat peptide (data not shown). These results indicated that the penetrating activity is remarkably decreased as a consequence of random conversion of amino acids within the Tat transduction domain. In addition, we made the PSIF-fused peptide library after fourth round panning (Fig. 3). In screening this peptide library, a tryptophan-rich (GSSSWWQRWWPPW) peptide was identified (Table 2). However, this peptide did not exhibit cytotoxicity when recombined with PSIF. This result indicated that this tryptophan-rich peptide binds to the cell membrane but does not penetrate through to the cytoplasm. Next, we reconfirmed that fixation of the cells significantly affected the cellular distribution of peptides (Fig. 4). 435B peptide (GPFHFYQFLFPPV) and 439A peptide (GSPWGLQHHPPRT) showed internalization characteristics similar to those of the parent Tat peptide. These FITC-labeled peptides did not show cytotoxicity at a dose of 10 μM. However, the two another clone (434C and 436C peptide) does not TAT-derived peptide , which is not consist of 13 amino acid or occur the flameshift, respectively. So we were excluded these two clones from followed experiment.

Table 2. Random Tat Peptide Library Sequence after 4th Panning

Clone	Sequence
1	GPMESLQAFWPPW
2	GSSSWWQRWWPPW
3	GSSSWWQRWWPPW
4	GVFLLKQVPQPSH
5	GSSSWWQRWWPPW
6	GRLWWLQLFEPGH
7	GLRKVPQSVPPDM
8	GSSSWWQRWWPPW
9	GHFLKPQVLRPTR
10	GQFMMRQYWPPVH
11	GSSSWWQRWWPPW
12	GSSSWWQRWWPPW
13	GSSSWWQRWWPPW
14	GLLKYQQWASPLC
15	GYFWYDQPWQPEQ
16	GRNHYIQRDNPVS
17	GVFHVLQNAIPQY
18	GSSSWWQRWWPPW
19	GTMPNMQHHDPAR
20	GSSSWWQRWWPPW
21	GSSSWWQRWWPPW
22	GSSSWWQRWWPPW
23	GTRYLVQYLFPHL
24	GRPATQQGLTPAR
25	GYIGTYQQWNPPP
26	GSSSWWQRWWPPW
27	GSSSWWQRWWPPW
28	GSSSWWQRWWPPW

Uptake of FITC-Labeled Peptides into Human and Murine Cells To address the question of whether 435B and 439A peptides were more active than the parent Tat peptide, peptides conjugated to FITC were constructed. Cellular uptake of both peptides were judged by flow cytometric analysis on human carcinoma A431 and Hela and CHO cells. Assuming that the surface-adsorbed 435B and 439A peptides were susceptible to tryptic degradation, we washed the cells five times with PBS and treated them with trypsin prior to assessing the amount of the internalized peptide. On A431

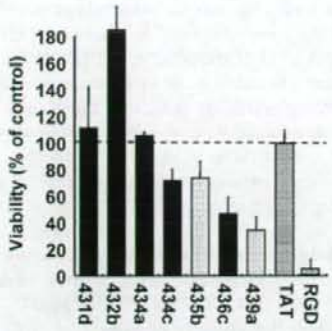

Fig. 4. Evaluation of Cell Penetrating Activity of Individual Clones

Cell penetrating activity was reconfirmed using the same method as that referred to in Fig. 3. Cell viability in response to fusion proteins was compared with that exhibited by exposure to the parent Tat13 peptide (cell viability=100%), and by exposure to 200 μg/ml cycloheximide (CHX; cell viability=0%).

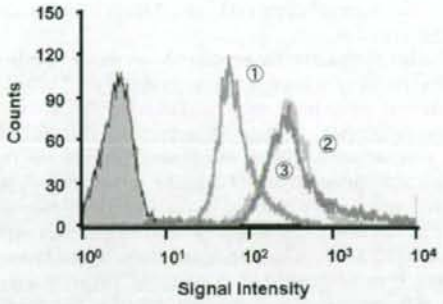

Fig. 5. Intracellular Penetrating Activity of FITC-Labeled CPPs on A431 Cells

Ten micromolar FITC-labeled 439A peptide (①), 435B peptide (②) and parent Tat peptide (③) were added to A431 cells. Gray area showed the distribution of non-treated cells. Following trypsinization, the quantity of penetrating peptide was evaluated in cells according the level of fluorescence intensity.

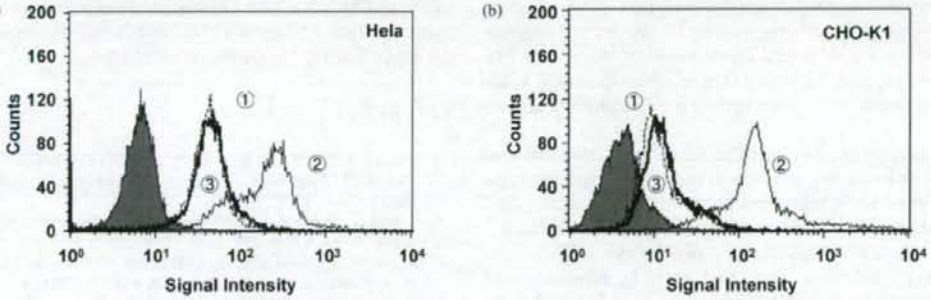

Fig. 6. Intracellular Penetrating Activity of FITC-Labeled CPPs on Human and Murine Cell Lines

Ten micromolar FITC-labeled 439A peptide (①), 435B peptide (②) and parent Tat peptide (③) were added to Hela (a) and CHO (b) cells. Gray area showed the distribution of non-treated cells. Following trypsinization, the quantity of penetrating peptide was evaluated in cells according the level of fluorescence intensity.

cells, the efficiency in terms of cell penetration between the parent Tat peptide and 435B peptide was almost the same, though the penetrating activity of the 439A peptide was decreased about 10 fold compared to the parent Tat peptide and 435B peptide (Fig. 5). However, on Hela and CHO cells, the efficiency of cell penetration between the parent Tat peptide and 439A peptide was almost the same, though the penetrating activity of the 435B peptide was increased about 10 fold compared to the parent Tat peptide and 439A peptide (Figs. 6a, b).

Inhibition of Endocytic Internalization Several studies were done to investigate the involvement of macropinocytosis or caveolae/raft-dependent endocytosis on peptide transduction domain such as TAT. The effect of the specific macropinocytosis inhibitor, amiloride, on TAT peptide penetration was determined. As seen in Fig. 7, treatment with amiloride did not inhibit 435B and 439A pepetration. Additionally, Methyl-β-cyclodextrin (MβCD)-sensitive caveolae/raft-dependent endocytosis of 435B and 439A peptides was detected, internalization of 435B and 439A *via* transduction is significantly affected by MβCD treatment.

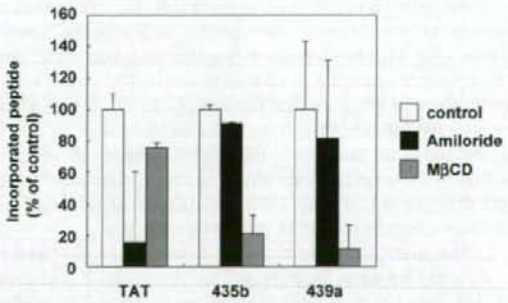

Fig. 7. Inhibitory Effects of Amiloride and MβCD for Peptide Incorporation on A431 Cells

FITC-labeled 435B peptide and 439A peptide were added to cells after amiloride and MβCD pre-incubated. The quantity of penetrating peptide was evaluated by FACS analysis according the mean of fluorescence intensity.

DISCUSSION

In an effort to search for novel CPPs, we have screened an M13 peptide phage display library comprised of CPPs based on the Tat transduction domain. From this screening approach, we have identified peptides that displayed a capability for cell penetration. In particular, a screen using A431 cells resulted in identification of peptide 435B and 439A that is able to penetrate cultured cells *in vitro* efficiently. Thus, this method of screening for CPPs using phage peptide li-

braries can be readily applied to select from a wide variety of possible peptides.

Whether the parent Tat peptide shows the penetrating capability of other known CPPs is uncertain.[12,16] The RGD peptide has long been used to facilitate the transport of bioactive molecules through adsorptive endocytosis.[17] However, comparison of short oligolysine peptides to that of equivalent length polymers of arginine showed that oligoarginine was much more efficient in carrying GFP into cultured cells.[18] Conversely, the ability of short oligolysine peptides, from 6 to 12 residues in length, has been shown to be more efficient than oligoarginine in carrying larger macromolecules (60—500 kDa) into cultured cells. In this study, the 435B and 439A peptides contained hardly any basic amino acids but still displayed an ability to mediate cell penetration more efficiently than the parent Tat peptide. This result indicated that the cell penetrating activity of CPPs is controlled not only by the electrical charge but also by the structural characteristics. Practically, it is known that the amino acid component and associated tertiary structure of peptides are essential for cell penetrating activity.[19] We did not examine our peptides on a structural level; however, the results presented here suggest the importance of hydrophobicity, as hydrophobic amino acids were enriched by sequential biopanning.

It has been theorized that the ionic interaction between positively charged Arg residues in these CPPs and the negatively charged phosphate head group of the membrane lipid bilayer plays a key role in CPP membrane interaction.[20] However, the exact mechanism by which these CPPs operate is still largely unknown. Work performed by several investigators has shown that Tat binds heparin and that this heparin/Tat interaction involves the basic domain of Tat.[21] Meanwhile, previous study showed that the histidine residues of peptide sequence might enhance an endosomal escape of the cargo.[22] In this study, the 435A and 439B peptides did not show enrichment of basic amino acids, but did exhibit an increase in proportion of hydrophobic amino acids. Several numbers of histidine residues were included in 435B and 439A peptide sequence compared to native TAT peptide. Accordingly, it is possible that these peptides do not penetrate through the binding of cell surface receptors such as HSPG but escape from endosome efficiently. Whereas the conformation of these peptides should be examined, our results suggest that the 435B and 439A peptides penetrate the cell membrane independently of cell surface receptor.

In this study, the transduction efficiency was observed to be different between the peptides fused with PSIF and those labeled with FITC. We think there are two ideas to explain this discrepancy. Firstly, the molecular size and structure of the respective cargo is different. Secondly, there is some possibility that the intracellular kinetics is different between the parent Tat peptide and our peptides. It is thought that the parent Tat peptide is transferred to nuclei after penetrating the cell membrane, while our mutant peptide-PSIF conjugate diffuses throughout the cytoplasm. We are currently examining the intracellular kinetics of these peptides in an effort to resolve this issue.

In this study, our peptides have a unique sequence compared to preexisting CPPs. These peptides are able to introduce a large molecule into the intracellular space more efficiently than the parent Tat peptide, the latter which is known to have a high level of transduction ability. Using these peptides, efficient introduction of large molecules to the cytoplasm is accomplished. As such, one could readily conceive of using these peptides to target disease-related proteins, revealed from extensive-omic analysis. Furthermore, our peptides can be used as analytical tools to explore the mechanism(s) of peptide penetration.

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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-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).

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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,