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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

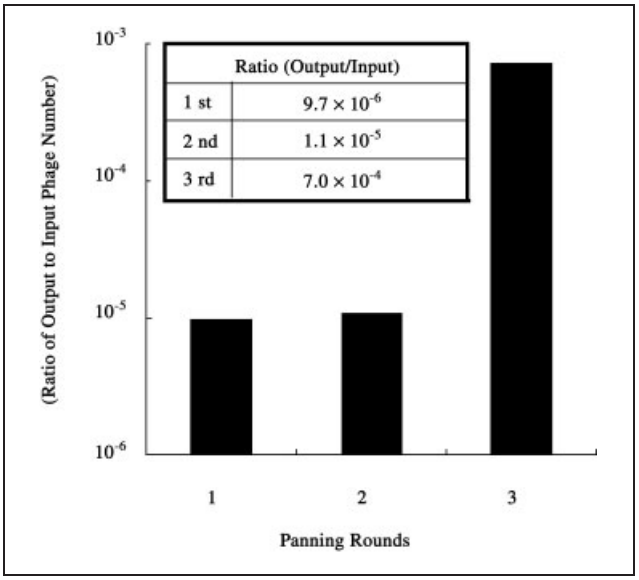

Fig. 1: Selection of binding and internalized phage clones by panning to A431. Phage clones binding to A431 cells with high affinity were collected. The ratio (output phage/input phage) in 3 rounds of panning was calculate. The number of phage clones binding to A431 cells increased with successive rounds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

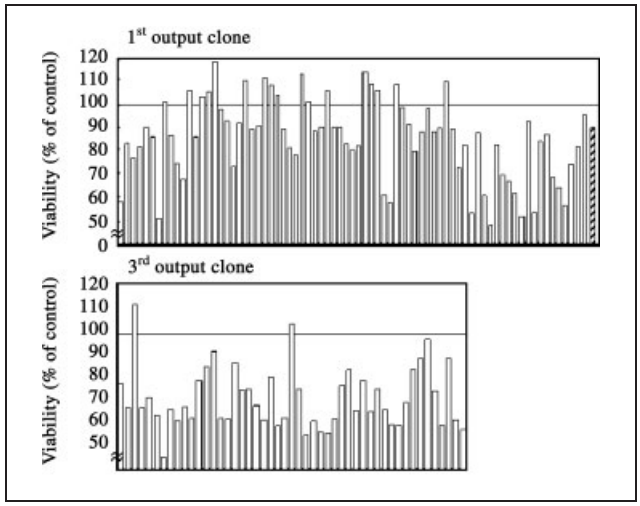

Fig. 2: Cytotoxic activity of randomly selected clones from a random 18mer peptide library. Phage clones were collected after 1 or 3 rounds of panning and genes encoding peptides were recombined with PSIF expression vector. Cytotoxicity mediated by PSIF fusion peptide from randomly selected clones was 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

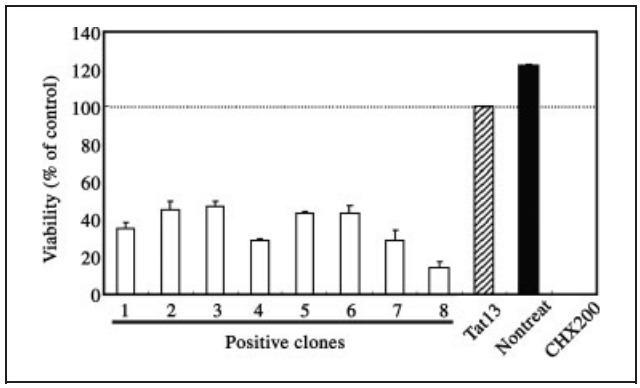

Fig. 3: Cytotoxic activity of positive clones. Positive clones were evaluated with TAT13-PSIF. Eight clones showing strong cytotoxic activity were identified and cytotoxic activity was measured by MTT assay. The viability of A431 cells treated with PSIF fusion TAT peptide was 100%, and the viability 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

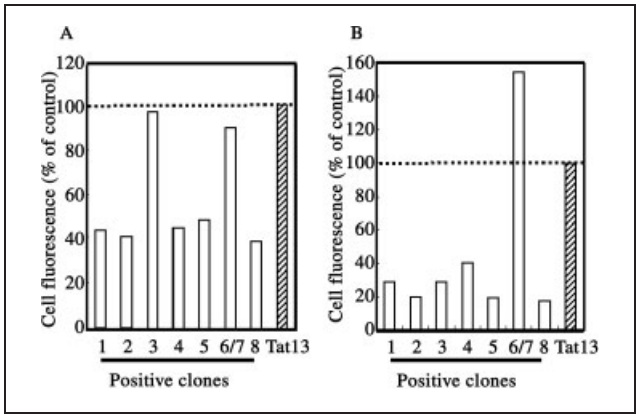

Fig. 4: Cellular uptake of positive clones in the cells. Positive clones were evaluated with FITC-labeled Tat13. A431 cells (A) or HeLa cells (B) were incubated with edium containing FITC-labeled peptide (1 mM) for 3 h. Intracellular translocated peptides were quantified with a a FACScan flow cytometer. 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)

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 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.

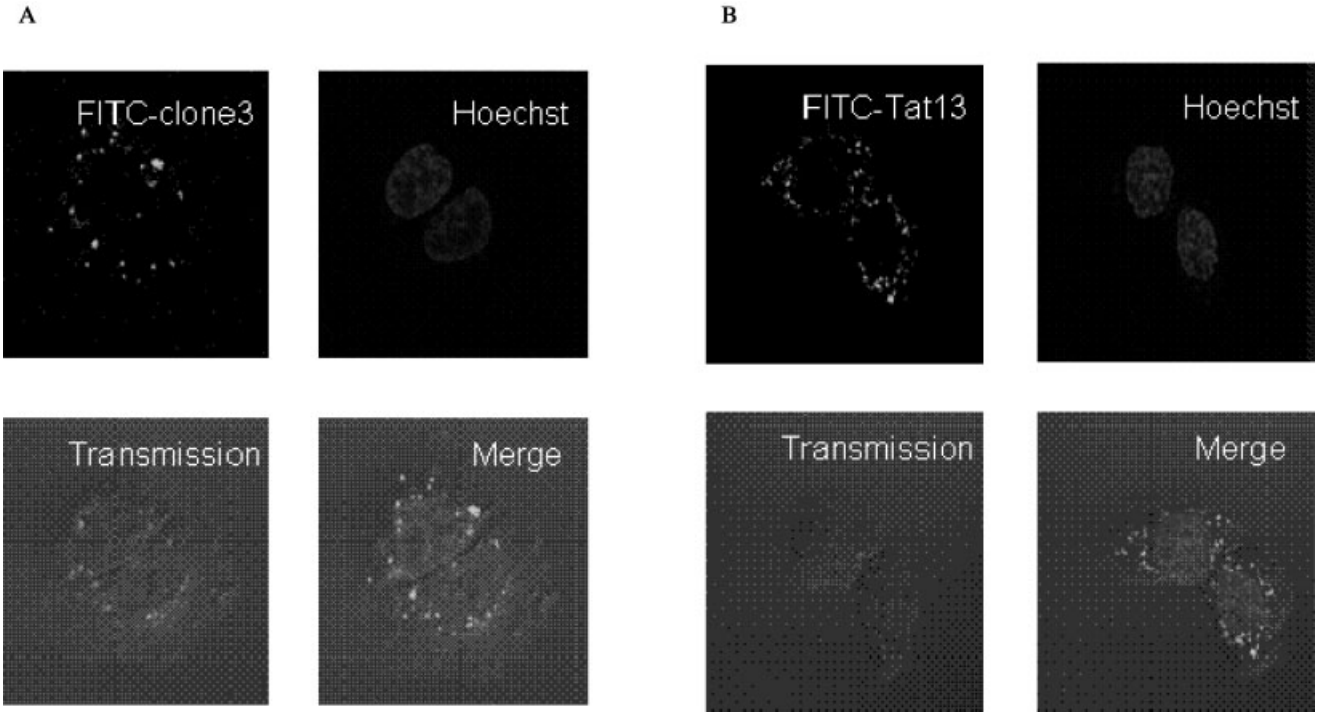

Fig. 5: Intracellular distribution of clone3 in living cells. A431 cells were cultured with FITC-labeled clone3 (A) or TAT peptide (B) for 3 h. Cells were washed and nuclei were stained with Hoechst 33342. Cells were examined by confocal laser microscopy

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′-GATTACGCCAAGCTTTGGAGCCTTTTTTTTGGAGATTTTCAACGTG-AAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTATGCGGC-CCAGCCGGCCATGGCC-3′), P-oligo4(5′-CGGCGCACCTGCGGCCGC-SNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNS-NNSNNSNNGGCCATGGCCGGCTGGGCCGCATAGAA-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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