Bioorganic & Medicinal Chemistry 21 (2013) 2560–2567

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Systematic screening of the cellular uptake of designed alpha-helix peptides

Kenji Usui [a,b,c,*,†], Takuya Kikuchi [a,†], Masayasu Mie [b], Eiry Kobatake [b], Hisakazu Mihara [a,*]

[a] Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan
[b] Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan
[c] FIRST (Faculty of Frontiers of Innovative Research in Science and Technology), FIBER (Frontier Institute for Biomolecular Engineering Research), Konan University, Kobe 650-0047, Japan

ARTICLE INFO

Article history:
Received 14 December 2012
Revised 16 February 2013
Accepted 18 February 2013
Available online 27 February 2013

Keywords:
Peptides
Library
Cellular permeation
Cellular engineering

ABSTRACT

The cellular penetration (CP) activity of functional molecules has attracted significant attention as one of the most promising new approaches for drug delivery. In particular, cell-penetrating peptides (CPPs) have been studied extensively in cellular engineering. Because there have been few large-scale systematic studies to identify peptide sequences with optimal CP activity or that are suitable for further applications in cell engineering, such as cell-specific penetration and cell-selective culture, we screened and compared the cellular uptake (CU) activity of 54 systematically designed α-helical peptides in HeLa cells. Furthermore, the CU activity of 24 designed peptides was examined in four cell lines using a cell fingerprinting technique and statistical approaches. The CU activities in various cells depended on amino acid residues of peptide sequences as well as charge, α-helical content and hydrophobicity of the peptides. Notably, the mutation of a single residue significantly altered the CU ability of a peptide, highlighting the variability of cell uptake mechanisms. Moreover, these results demonstrated the feasibility of cell-selective culture by conducting cell-selective permeation and death in cultures containing two cell types. These studies may lead to further peptide library design and screening for new classes of CPPs with useful functions.

1. Introduction

Significant advances in understanding the intracellular behavior of biomolecules in vital phenomena have opened up the possibility of controlling cellular events using artificial functionalized molecules. These molecules, however, are often limited by an inability to enter cells, thereby reducing their application potential.[1,2] The study of the cellular internalization of such molecules is therefore one of the most important fields of chemical biology. A number of successful strategies have been proposed so far, including microinjection, electroporation, the use of viral and nonviral vectors, and lipid encapsulation. However, these strategies still have some drawbacks, such as inefficient drug delivery, cellular damage and toxicity, limitations in cell targeting or organelle targeting, and restrictions based on drug and cell type, that have made it difficult to expand their biological applications. Cell-penetrating peptides (CPPs)[3–11] have been studied extensively and are considered one of the most promising methods for the cellular internalization of biologically active molecules. CPPs can rapidly deliver large molecules into any cell type,[12] and because they are short (10–30 residues) cationic peptides, they are easily synthesized. Additionally, CPPs can easily be attached to bioactive molecules and allow these molecules to maintain their activities after uptake. CPPs can be divided into three classes according to their origin.[9,13] The CPPs in the first class are derived from natural proteins, including the TAT peptide. The second class of CPPs, which includes transportan and arginine-rich peptides, are modified versions of the first class of CPPs. A novel third class of CPPs has been identified by the screening of random phage display libraries or synthetic peptide libraries for cell-penetration (CP) properties. There have been some large-scale systematic attempts to identify sequences with optimal CP properties in the first and second classes.[14–16] The third class of peptides remains poorly characterized,[17] especially by comparison of various sequences with systematical replacement of amino acids. Although many excellent studies of CPPs have been performed to date, the novel class of CPPs is especially promising due to their higher cellular specificity[18] or multifunctionality, including cell type-specific cytotoxicity and organelle targeting,[19] which may broaden their applications in cell and tissue engineering. Alternatively, CPPs can be characterized into two groups

* Corresponding authors. Tel.: +81 78 303 1418; fax: +81 78 303 1495 (K.U.); tel.: +81 45 924 5756; fax: +81 45 924 5833 (H.M.).
E-mail addresses: kusui@center.konan-u.ac.jp (K. Usui), hmihara@bio.titech.ac.jp (H. Mihara).
† These authors contributed equally to this work.

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.02.030

according to their chemical properties. CPPs in the first group are short peptide sequences that are rich in basic amino acids, including arginine-rich peptides.[14] CPPs in the second group are amphiphilic in nature: that is, they include both hydrophilic (mostly cationic) and hydrophobic amino acids. However, it remains unclear which types of sequences exhibits a better CP properties and how the CP activity depends on peptide properties, such as charge, hydrophobicity or α-helicity. CP analyses using systematically designed peptides would be useful to address these questions and improve CPP functions, such as cell-specific targeting.

In this paper, we performed systematic screening of synthetic amphiphilic α-helix peptide candidates for the novel third class of CPPs (the artificial peptides). Basically, amphiphilic cationic α-helical peptides including antimicrobial peptides have membrane perturbation activity and often have cell death activity. Consequently, we would be able to easily find relatively low toxic peptides or cell-selective toxic peptides having membrane permeation activity from these peptides. We expect that these peptides will offer the following improvements over current CPPs: (i) short-peptide libraries are easy to design and systematically synthesize, with a cationic amphiphilic α-helical structure suitable for permeation; (ii) systematic analyses based on peptide libraries can be used to screen for permeation properties in various cell types, and cell-specific permeating sequences could be identified; and (iii) because few studies comparing the cellular uptake of designed synthetic peptides have been reported to date, de novo designed peptide sequences with high functionality or multifunctionality could be found.

We systematically designed libraries of peptides composed of various secondary structures, including α-helices,[20–24] and analyzed them using protein microarrays.[25–30] In our array technique, the obtained binding response pattern, regarded as a 'protein fingerprint' (PFP), is used to establish the identity of a target protein and correlate the recognition properties of a target protein to library peptides.[20–24,31] Then, by applying statistical analyses, such as hierarchical clustering analysis (HCA) and principal component analysis (PCA), to PFPs we can draw high-confidence correlations between the target proteins and their biological function, based primarily upon peptide charge and hydrophobicity data[22,24] (HCA, which is generated from Euclidean distances, provides similarity information among PFPs. PCA is a linear mathematical technique to find base vectors [principal components (PCs)] that expand the PFP space, and can be applied to understand what major contributors distinguish the proteins by the PFP data. PCA reduces the number of variables (features) to a more manageable size). We then adapted the designed peptide array system for use in cellular studies. Using a strategy similar to that used for protein identification and characterization via PFP, cell fingerprints (CFPs) can be generated from patterns of cellular responses upon stimulation with library peptides, revealing how peptide properties affect a particular type of cell.[32]

In this study, we systematically assayed the uptake of synthetic α-helical peptides in four cell lines using the CFP technique and statistical analyses. Confocal fluorescent microscopy was employed for these detailed assays because confocal fluorescent microscopy offered exclusion of fluorescence from membrane-adsorbed peptides and could show fluorescence only from the peptides inside a cell. This study is an unprecedented systematic and comprehensive study of cellular uptake (CU) activity that addresses peptide library design and screening for novel CPPs with increased specificity and increased functionality in particular cells. Furthermore, we demonstrated an application of these synthetic designed peptides in cell-selective culture, expanding the potential use of permeating peptides in cell and tissue engineering.

2. Results and discussion

2.1. CU assay in HeLa cells using the designed α-helical peptide library

We optimized a CU assay procedure in human cervical carcinoma (HeLa) cells using peptides from an α-helical peptide library in which we systematically replaced amino acid residues with other residues of varying charge and/or hydrophobicity. The strategy used for the α-helical peptide library construction is shown in Figure 1. The peptide LKKLLKLLKKLLKL[33] (L8K6; No. 000), which binds to calmodulin in the presence of Ca^{2+} and has antimicrobial activity,[34] was used as the mother cationic amphiphilic α-helical peptide. The length of this sequence could provide not only ease of design and synthesis of peptides but also various systematical replacements of amino acid residues remaining α-helical structure. We designed peptides in which four residues in the central region of L8K6 and LEKLLELLKELLEL (L8K2E4, No. 201) were replaced with other amino acids.[23] In this assay, we selected 54 peptides from this library (Table S1) in the following manner: (1) to dynamically change the charge of the hydrophilic face of the helix, residue Z_1 was replaced with Glu or Arg; (2) to change the hydrophobicity of the hydrophobic face of the helix, X_2 was replaced with Ala or Phe; (3) to vary the aromatic character, hydrophobicity and charge of the helix boundary between the hydrophobic and hydrophilic faces of the helix, X_1 and Z_2 were replaced with Trp, Ile, Ala, Arg, His, Glu, Gln, Thr, or Tyr. Additionally, the fluorophore 5(6)-carboxytetramethylrhodamine (TMR) was introduced at the N-terminus of each peptide in the library to allow the detection of CU activity using fluorescent confocal microscopy.

The cells were incubated with each peptide (2 μM) for 1 h at 37 °C and then observed with confocal microscopy. Because the confocal microscopy offered exclusion of fluorescence from membrane-adsorbed peptides and could show fluorescence only from permeating peptides, the ability of each peptide to permeate cells

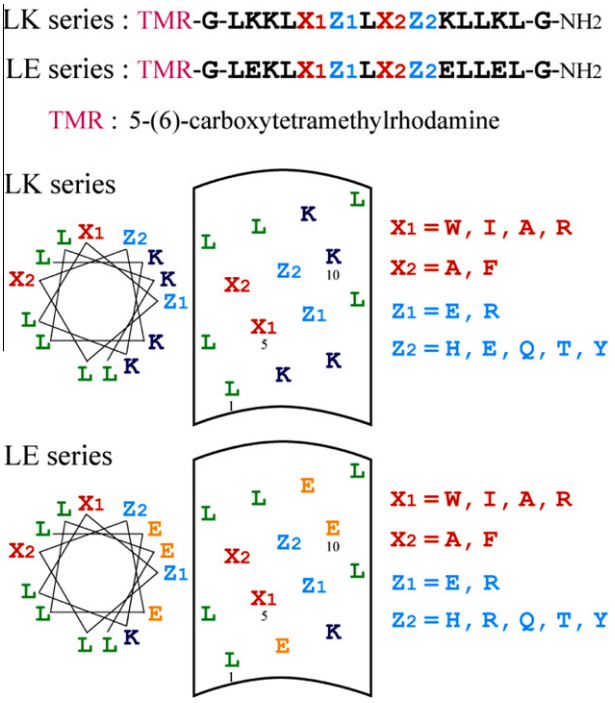

Figure 1. Peptide library strategy in this study. Strategy used in the construction of the library consisting of 202 α-helical peptides.[23] We designed peptides in which four residues (X_1, Z_1, X_2, and Z_2) in the central region of LKKLLKLLKKLLKL (L8K6; No. 000) and LEKLLELLKELLEL (No. 201 L8K2E4) were replaced with other amino acids.