Functional Peptides That Target Biomembranes: 
Design and Modes of Action

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Biomembranes are important targets in molecular design. Our laboratory has been exploring the design of functional peptides that modulate membrane barrier function, lipid packing, and structure. Evaluation of the results obtained and analyses of cellular mechanisms have yielded peptides with more refined designs and functions. This review highlights the progress made in our laboratory towards the development of unique peptides that modulate membrane properties.

Key words peptide; cell membrane; intracellular delivery; curvature; endocytosis; antibody

1. Introduction

The creation of biofunctional molecules is a challenge at the interface of chemistry and biology. Peptides are versatile molecules in molecular design. Our accumulated knowledge of protein structure and activity allows for the design of peptides that form specific structures to provide a desired function within a given environment. Synthetic peptides can be made to include non-proteinogenic amino acids or functional units, resulting in unique properties that are not observed in natural systems.

The cell membrane (plasma membrane) serves as a barrier between the intra- and extracellular environments. Cellular components, including proteins, nucleic acids, and electrolytes, can be contained safely within a cell, thereby ensuring cellular homeostasis and proper cellular activity. However, difficulties can arise when trying to deliver hydrophilic proteins or other bioactive molecules into cells. Mammalian cells also contain various vesicular compartments, such as endosomes, lysosomes, mitochondria, the endoplasmic reticulum, and the Golgi apparatus, which are protected by surrounding membranes. Therefore, control of membrane structure and permeability is an attractive approach for modulating cellular activity. Membrane permeability is particularly important when considering cytosolic delivery of biomacromolecules. Excessive permeabilization of the cell membrane is accompanied by leakage of cellular components that can severely damage cells. In addition, a cell membrane is a “soft” barrier. Membrane structures have to change flexibly to facilitate various cellular events, including cell growth, movement, division, and nutrition uptake. Mechanosensing and biosignaling via tension changes in the cell membrane can elicit cellular events. Therefore, the acceleration or suppression of changes in cell membrane structure, or the modulation of membrane tension, may be pathways to achieving control over cellular events. The opportunities available to achieve such control via molecular design are vast. Structural alteration of membranes is generally accompanied by changes in membrane curvature and possibly membrane tension, which can, in turn, affect lipid packing. Efforts to modulate membrane structure would therefore help us understand the relationships between each of these factors and subsequent cellular events.

Our research group focuses on peptide interactions with membranes. We have designed and developed functional peptides that interact with membranes to create unique and efficient cytosolic delivery systems or peptide tools that can be used to modulate cellular structures and activities. Some of these accomplishments are highlighted in this review.

2. Membrane Permeability of Arginine-Rich Peptides 
and Modes of Action

2.1. Membrane Permeability of Arginine-Rich Peptides

While there are numerous means of delivering genes and nucleic acids into cells, there are relatively few ways to deliver peptides and proteins. To this end, a basic peptide derived from the human immunodeficiency virus (HIV)-1 trans-activator of transcription (Tat) protein (positions 48–60) reportedly delivers peptides and proteins into cells while maintaining their expected activity. Importantly, this peptide is involved in transcription regulation and not in the invasion of host cells. Positions 48–60 correspond to the RNA-binding region of the protein and contain a high proportion of basic amino acids (GRKRRQRRRRPQ, referred to as TAT in this review). In addition, this sequence is assumed not to have a helical structure. Although hydrophilic conjugates of this sequence with various peptides and proteins are translocated into cells where they maintain their expected bioactivities (Fig. 1). Cellular internalization of TAT at 4 °C has also been reported, excluding the involvement of endocytosis (energy-driven cellular machinery requiring ATP) in
the internalization.\textsuperscript{1} In addition, several peptides have been reported to achieve cytosolic delivery of bioactive but membrane-impermeable molecules of interest via conjugation or complex formation, and these peptides are collectively named cell-penetrating peptides (CPPs).\textsuperscript{2–5}

Elucidating the modes of cellular internalization of TAT would contribute significantly to the design of transmembrane delivery systems. Assuming the presence of a specific receptor for TAT, D-amino acid versions of TAT peptide (dTAT) and R9-TAT, in which amino acids other than arginine are replaced with arginine, may not serve as a ligand \textsuperscript{6} (Table 1). However, all of these peptides exhibited similar internalization characteristics as the TAT peptide. This suggests the importance of arginine in the internalization of peptides. Indeed, lysine-rich sequences derived from nuclear localization signal sequences of simian virus 40 (=PKKKRKV) and nucleoplasmin (=KRPAIKKAGQAKKKK) did not exhibit marked internalization. Since TAT is contained within the RNA-binding sequence of the Tat protein, ten peptides corresponding to RNA-binding segments of other proteins were synthesized and evaluated for their ability to enter cells. Surprisingly, all but one of the tested peptides, human U2AF with only three arginine residues, were internalized.\textsuperscript{6} Arginine-rich peptides from the DNA-binding sequences of three transcription regulators were also internalized. These results suggest that, regardless of their origins, peptides rich in arginine can be transported across cell membranes. We therefore prepared a series of oligoarginine peptides comprising 4, 8, 12, and 16 arginine residues. Peptides of 8 and 12 residues were efficiently internalized into cells.\textsuperscript{6} These data suggest that there is an optimal number of arginine residues for internalization. The importance of arginine- or guanidine-containing functional groups has also been proposed independently by Wender and colleagues.\textsuperscript{7,8}

### 2.2. Involvement of Macropinocytosis in Endocytic Uptake of Arginine-Rich Peptides

As noted in section 2.1, it was initially thought that endocytosis was not involved in the cellular internalization of TAT and related peptides. However, later studies revealed that membrane permeabilization accompanied by fixation (employing organic solvents or reagents) leads to re-distribution of cell-membrane-adsorbed and endosome-trapped arginine-rich peptides into the cytosol and nucleus.\textsuperscript{9} Reevaluation of the mechanisms controlling the internalization of TAT and other arginine-rich peptides revealed that endocytosis does, in fact, play a role in the internalization

![Fig. 1. Conjugation or Stable Complex Formation with Arginine-Rich, CPPs Facilitates the Delivery of Proteins of Interest (Cargo) into the Cell Interior, i.e., Cytosol](image)

| Table 1. Examples of Arginine-Rich Peptides Showing Membrane Permeability |
|------------------|------------------|------------------|------------------|
| TAT and its analogs | GRKRRQRRRRPPQ | Grkrrqrqqrrppq\textsuperscript{a} | RRRRRRRRRPPQ |
| Other RNA-binding peptides | HIV-1 Rev (34–50) TRQARRRRRRRWRERQR | FHV Coat (35–49) RRRRRTRRRRRRRVR | BMV Gag (7–25) KMTRARQQARARWNRTAR |
| | HTLV-II Rex (4–16) TRRQRRRARRNR | Oligoarginines | Rn (n = 6–12) Rn |

\textsuperscript{a}Lowercase letters represent D-amino acids.

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**Biography**

Shiroh Futaki was born in 1959 in Kanazawa in the Ishikawa Prefecture of Japan. He received his bachelor’s degree in Pharmaceutical Science from Kyoto University in 1983 and his Ph.D. from the same University in 1989 under the supervision of Professors Haruaki Yajima and Nobutaka Fujii. He was appointed as a Research Associate of the Faculty of Pharmaceutical Sciences at the University of Tokushima in 1987 (Professors Tadashi Akita and Masahito Ochiai) and as an Associate Professor of the same Faculty in 1992 (Professor Mineo Niwa). In addition, he spent 16 months (1989–1991) in the United States as a Postdoctoral Associate in the Department of Biochemistry at Rockefeller University with Professor James M. Manning. He was appointed as an Associate Professor at the Institute for Chemical Research at Kyoto University (Professor Yukio Sugiura). He has been a Professor of Biochemistry at the current institution since 2005. His research interests include the design of functional peptides, especially those that interact with biomembranes. He received The Japanese Peptide Society Award for Young Scientists and Daiichi Pharmaceutical Award in Synthetic Organic Chemistry, Japan, in 1997, The Pharmaceutical Society of Japan Award for Young Scientists in 1998, and The Pharmaceutical Society of Japan Award in 2020. He was invited as a Visiting Professor by Université Pierre et Marie Curie, France, in 2010. He has been an Honorary Member of the Hungarian Academy of Sciences since 2019.
of these peptides.

Macropinocytosis is an actin-driven, fluid-phase endocytosis process. The role of macropinocytosis in the endocytic uptake of arginine-rich CPPs has been reported by our group and others. The presence of a macropinocytosis inhibitor, such as ethylisopropyl amiloride, significantly decreased the cellular uptake of arginine-rich peptides. Polydextran (70 kDa) has been used as a marker of macropinocytosis to show the bulk engulfment of solutes in macropinocytosis-induced cells. Cellular treatment with arginine-rich peptides also increased the uptake of 70-kDa polydextran.

Arginine-rich CPPs have a strong affinity for membrane proteins decorated with sulfated saccharides (membrane-associated proteoglycans), resulting in their accumulation on cell surfaces (Fig. 2). This leads to reorganization of actin and membrane ruffling, following activation of cellular Rac-1 protein. Eventual membrane fusion leads to the formation of large endosomes, or macropinosomes. The diameter of a macropinosome often exceeds 1 µm, which is significantly larger than endosomes created in clathrin- and caveolae-mediated endocytosis (typically, 120 and 80 nm, respectively). Arginine-rich peptides had little effect on the induction of macropinocytosis in cells deficient of membrane-associated proteoglycans. This suggests that membrane-associated proteoglycans play an important role in macropinocytosis induction.

Using photo-crosslinking studies to find the receptors responsible for inducing macropinocytosis via interactions with arginine-rich peptides, we identified CXCR4 as a macropinocytosis induction receptor for dodecaarginine (R12). CXCR4 is a chemokine receptor and plays a role in the HIV infection of host cells. Its inherent ligand, stromal cell-derived factor (SDF)-1α, and the gp120 envelop glycoprotein of HIV-1 were also found to induce macropinocytosis.

Although the cell-surface adsorption of arginine-rich CPPs and massive endocytic uptake by macropinocytosis are important factors determining the efficiency of intracellular delivery using CPPs, the exact mechanism of their translocation from endosomes into the cytosol, i.e., endosomal escape, remains unclear. It has been suggested that bis(monoacylglycero)phosphate, an endosome-specific lipid, plays a major role in this process. However, further study is required for a full understanding of the mode of cytosolic translocation toward the design of more effective delivery systems.

2.3. Translocation of Arginine-Rich Peptides through Cell Membranes Although the involvement of endocytosis, including macropinocytosis, has been suggested for entry of arginine-rich peptides into the cytosol, detailed studies have shown that, depending on the conditions, arginine-rich peptides are able to pass through cell membranes without notable damage to the membrane. We examined the mechanisms of cell internalization using fluorescently labeled R12. Confocal laser scanning microscopy showed punctate, dot-like signals corresponding to fluorescently labeled R12. However, once the R12 concentration exceeded a certain threshold, the direct influx of R12 into the cell interior was predominant, accompanied by diffuse cytosolic labeling by fluorescently labeled R12 (Fig. 3). Notably, cell membranes under these conditions maintained their integrity with no discernable membrane rupture. Similar methods of cell entry have been observed with other arginine-rich peptides. Therefore, certain levels of cell-surface accumulation may drive the arginine-rich peptides through the cell membrane without endocytosis. This method of cell entry was observed even at 4°C where endocytosis, an energy-driven cellular activity, is non-operational. The importance of peptide hydrophobicity in membrane translocation was proposed in a later study. The attachment of appropriate hydrophobic amino acids or acyl moieties better facilitated the membrane translocation of arginine-rich peptides.
a translocation catalyst.28–30) This approach is applicable to chemical biology and biophysics studies and has led to the first intracellular NMR analysis of stable isotope-labeled proteins in mammalian cells.31)

We recently demonstrated the importance of lipid packing in the membrane translocation of arginine-rich peptides.32) It has been hypothesized that the guanidino group of arginine forms ion pairs and hydrogen bonds with the phosphate in the head group of phospholipids, a major component of the cell membrane.3,33) These bonds stabilize the interactions between arginine-rich peptides and the membrane. However, cell-surface adsorption of the peptide does not necessarily lead to membrane translocation. If the lipid packing is loose, then acyl groups of phospholipids in the membrane core are more exposed to the cell surface, allowing for greater interaction between the peptide backbone and the membrane core, facilitating peptide translocation. We found that loosening lipid packing via pyrenebutyrate treatment accelerated the cell-membrane translocation of arginine-rich peptides.32) In addition, the preferential influx of arginine-rich peptides through membrane areas with looser lipid packing has been observed.32) Therefore, lipid packing is another possible factor in the membrane translocation of arginine-rich CPPs.

2.4. Cytosolic Delivery Using Arginine-Rich Peptides

The ability and potential of arginine-rich peptides to facilitate cytосolic delivery has been demonstrated by our group and collaborators via conjugation or complex formation with molecules of interest. The conjugation of peptides and proteins with arginine-rich peptides, such as octaarginine (R8), allows them to be carried into cells while maintaining their expected bioactivities. N-terminal stearylation of R8 (R8), allows them to be carried into cells while maintaining their expected bioactivities. N-terminal stearylation of R8 facilitates complex formation with plasmids, facilitating gene delivery into cells at levels comparable to those obtained with lipofectamine, a widely used transfection reagent.34) Stearylated R8 allows for the facile decoration of liposomes and lipid nanoparticles with R8. Intracellular gene and small interfering RNA (siRNA) delivery via R8-modified liposomes was demonstrated by Harashima and collaborators.35,36) We also showed that D-forms of R8 tend to accumulate on cancer cells.37) It has been suggested that proteoglycans play a major role in this accumulation.

3. Peptides Affecting Membrane Curvature, Lipid Packing, and Membrane Tension

Membrane curvature, lipid packing, and membrane tension are closely related factors that determine membrane structure and fluidity, which have considerable effects on cellular behavior.38,39) For example, changes in membrane curvature accompany cell movements and rely on continuous changes in lipid packing and membrane tension. Membrane curvature is also a feature of endocytosis, accompanied by consecutive invagination and fusion of the cell membrane. Peptides that affect membrane curvature and tension may therefore be a tool for modifying cellular structure and function. Epsin is a cytosolic protein that is involved in membrane invagination and the formation of clathrin-coated pits.40,41) A peptide corresponding to the N-terminal 18 residue of epsin (EpN18) induces posi-
tive curvature in cell membranes. The induction of curvature in the membrane also loosens lipid packing.\textsuperscript{42} The membrane translocation of R8 was also promoted in the presence of EpN18 (Fig. 5). Trimerization of EpN18 enhances its ability to induce curvature and loosen lipid packing.\textsuperscript{43} A nine-residue amphiphilic peptide (R6W3) was also shown to stimulate endocytic uptake by inducing membrane curvature.\textsuperscript{44}

As an example of a peptide that reduces membrane tension, an amphipathic peptide derived from the influenza M2 protein (M2[45–62]) yields lamellipodia at multiple sites in the cell.\textsuperscript{45} M2[45–62] can inhibit cell motility, based on scratch wound migration and transwell migration assays. Increases in neurite outgrowth were also observed following treatment with M2[45–62].

4. Approaches for Delivering Macromolecules into Cells

4.1. Attenuated Cationic Amphiphilic Lytic Peptides for Cytosolic Protein Delivery

Although arginine-rich CPPs may serve as effective and efficient vectors for transmembrane delivery, perturbation of the membrane by such peptides may not be significant. This would result in low cytotoxicity upon delivery. However, more efficient methods based on different design philosophies are needed to stimulate endosomal escape and the cytosolic delivery of large macromolecules such as antibodies.

With regard of membrane perturbation, cationic amphiphilic peptides, which are often employed as antimicrobial or membrane-lytic peptides, may exhibit stronger membrane perturbation than arginine-rich peptides.\textsuperscript{46} The cell surface is thought to have a weak negative charge due to cell-surface proteoglycans and sialic acids. Cationic peptides are therefore easily adsorbed onto cell surfaces. Although both types of peptide effectively adsorb onto the cell surface, arginine-rich peptides exhibit considerably less membrane-lytic activity than cationic amphiphilic peptides due to the lack of hydrophobic moieties. Thus, effective systems for the cytosolic delivery of biomacromolecules may be constructed using highly lytic, amphiphilic cationic peptides.

Based on this concept, we developed a delivery technique using attenuated cationic amphiphilic lytic (ACAL) peptides.\textsuperscript{47} Cationic amphiphilic lytic peptides generally feature a hydrophobic face on one side of a helical surface with a cationic face on the other side. The substitution of amino acids on the hydrophobic face to negatively charged amino acids, such as glutamic acid (Glu), significantly decreases hydrophobic interactions and the ability of the lytic peptide to perturb the membrane. However, if the net hydrophobicity of the Glu-bearing peptide is maintained, then it may adsorb to the cell membrane and be endocytosed. Endosomal acidification can diminish the negative charge and allows recovery of membrane-lytic activity, allowing cytosolic translocation of the protein of interest (cargo).

Our group previously reported that R12 interacts with chemokine receptor CXCR4 to induce macroinocytosis.\textsuperscript{19} The ligand SDF-1\textsubscript{α} or envelop protein gp120 of HIV-1 can also induce macroinocytosis. Since the N-terminus segment of SDF-1\textsubscript{α} is involved in CXCR4 binding,\textsuperscript{54,55} we screened the N-terminal peptides for similar abilities. We found that the 21-residue peptide making up the N-terminus of SDF-1\textsubscript{α}
Macropinocytosis-inducing peptide (SN21)

(i) Induction of macropinocytic uptake

Cytosol

Membrane-lytic peptide (LK15)

(ii) Membrane lysis

Macropinosome

Fig. 7. Combination Strategy of Using a Macropinocytosis-Inducing Peptide with an Endosomolytic Peptide for Effective Cytosolic Delivery of Biomacromolecules

SN21 is a macropinocytosis-inducing peptide. (i) Administration of cargo in the presence of SN21-LK15 leads to macropinocytic uptake of cargo and SN21-LK15. (ii) Possible lysis of endosomal membranes by the action of LK15 results in leakage of cargo into the cytosol. (Color figure can be accessed in the online version.)

(SN21) has a strong ability to induce macropinocytosis.60 LK15 is an artificial membrane lytic peptide.57 The conjugate of SN21 with LK15 (SN21-LK15) is capable of delivering nucleic acids, e.g., plasmids, siRNA, and proteins such as Cre recombinase and immunoglobulin G, into cells (Fig. 7). Detailed mechanisms of cytosolic protein delivery using SN21-LK15 are now under study in our laboratory.

5. Conclusion

This review highlights accomplishments made in our laboratory on peptide-membrane interactions and their application to cytosolic delivery and cell manipulation. During the course of these studies, we discovered several new aspects of peptide-induced cellular response based on biophysical and biological perspectives. Macropinocytosis is one example and was previously thought of as a non-specific and relatively minor form of endocytosis. However, due to the involvement of macropinocytosis in the transport of various extracellular materials and its importance in nutrition uptake by cancer cells, there is a growing need to understand the underlying cellular machinery. Peptides are a fascinating tool for controlling and manipulating biological systems and developing associated applications. Unique concepts in molecular design and an understanding of the interplay between peptides and membranes are needed to fully explore the possibilities.

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