Advanced Studies in Biology, Vol. 5, 2013, no. 1, 11 – 25
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Primary Effectors in the Mechanisms of Transmembrane Delivery of Arginine-rich Cell-penetrating Peptides

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Abstract

The cell membrane is a biological boundary that separates the interior of cells from the extracellular environments. While small non-polar molecules readily cross cell membranes, the cell membrane precludes the passage of most macromolecules, including proteins, DNAs, and RNAs. However, basic cell-penetrating peptides (CPPs) can enter cells, and can co-transport an array of normally impermeable molecules into cells. In this report, we identified factors that determine the mechanisms of CPPs entry. Three arginine-rich CPPs (R9,

SR9,and HR9) were studied in live human, plant, and bacterial cells. Pharmacological and physical treatments were used to elucidate the nature of the transport mechanism. The route of internalization was relatively unaffected by cell type, but was dependent on the nature of the CPP as well as the nature of the transported cargo.

Keywords: Blue fluorescent protein (BFP), Cell-penetrating peptide (CPP), Direct membrane translocation, Energy-dependent Endocytosis, Green fluorescent protein (GFP), Macropinocytosis, Protein transduction domain (PTD)

Abbreviations: BFP, blue fluorescent protein; CPP, cell-penetrating peptide; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; GFP, green fluorescent protein; N/P, nitrogen/phosphate; NEM, *N*-ethylmaleimide; PBS, phosphate buffered saline; QD, quantum dot; R9, nona-arginine; SD, standard deviation; SR9, synthetic nona-arginine; Tat, transactivator of transcription

1. Introduction

The cell membrane, composed of a bilayer of phospholipids and proteins, provides a selectively permeable hydrophobic barrier that isolates the cell from the hostile extracellular environment. Membrane permeability depends on the size and polarity of substances, as well as the existence of specific intrinsic membrane transporters. In general, macromolecules, such as proteins and DNAs, are unable to cross cell membranes. Instead, various endocytic pathways, including lipid raft-dependent processes, provide the major routes for cellular entry of exogenous macromolecules.[1] Thus, the cell membrane is a gate that controls the movement of materials in and out of cells. The membrane is also involved in numerous cellular processes, such as signal transduction, cellular potential maintenance, nucleation of multiple protein complexes, and cytoskeleton anchoring. Transport of molecules across the membrane is quite complex as it is affected by the phospholipid, glycolipid, cholesterol, and protein composition of the membrane. Cellular entry processes are also species dependent.[1]

In 1988, transactivator of transcription (Tat) protein from the human immunodeficiency virus type 1 (HIV-1) was shown to be capable of overcoming the hydrophobic barrier of the plasma membrane during cellular internalization of molecules.[2,3] The domain in the Tat protein that mediates cellular entry contains 11 amino acids (YGRKKRRQRRR), and the number of basic residues was thought to correlate with its efficiency of membrane penetration.[4,5] Subsequently, short peptides derived from the key domain in the Tat protein that mediates membrane penetration were synthesized and tested. These peptides were termed cell-penetrating peptides (CPPs) or protein transduction domains (PTDs).[4] CPPs can be classified into three groups:

protein-derived peptides, model peptides, and designed peptides.[6] Tat is an example of protein-derived CPP. Peptides with repeat motifs or poly-residues are model CPPs. The amphipathic *peptide* MPG is an example of a designed CPP insofar as it is a chimeric protein containing domains from multiple peptides.[6] Studies have demonstrated that model CPPs containing nona-arginine (R9) residues are 20-fold more efficient at transduction.[7,8] Moreover, CPPs are efficient shuttles of cargoes, such as proteins, nucleic acids, nanoparticles, and liposomes. These cargoes maintain their biological activities after being transported into cells.[9,10] The size of the cargoes can be up to 200 nm in diameter.[11] The internalization kinetics of CPPs is rapid, with a first-order rate constant of 0.007 sec^{-1}, with no toxicity observed at concentrations up to 100 μM.[12]

Despite many studies using various biological and biophysical techniques, our understanding of the mechanisms of CPP entry remains incomplete and controversial. Low temperature reduced sulfated proteoglycans on cell surface that are involved in endocytosis, but did not stop the internalization of CPPs.[5] Their data further suggested that CPPs enter cells by an energy-independent pathway. Additional studies indicated that CPPs enter cells via clathrin-, caveolin-dependent endocytosis, or actin-dependent macropinocytosis.[13–20] Compositions of CPPs, types of cells, nature of cargoes, and organisms are factors that might influence the cellular uptake mechanisms.[13–20] Previously, we demonstrated that arginine-rich CPPs, such as R9, can deliver biologically active macromolecules into different kinds of cells and species following covalent, noncovalent, or combined covalent and noncovalent association.[21–38] Our data suggested that internalization of CPPs covalently linked with cargoes, such as R9-green fluorescent protein (GFP) fusion protein, was neither clathrin-dependent nor caveolae-dependent.[21] In certain types of cells, macropinocytosis was the major route of entry when CPP and cargo were associated noncovalently.[24–28] In some cases, penetration of CPPs with their cargoes was mediated by multiple pathways.[29,30,32,34]

In this report, we investigate transduction efficiency of various types of CPPs, cargoes, and types of cells from various organisms. Pharmacological and physical modulators of endocytosis were used to identify the molecular mechanisms of cellular entry. We identify multiple factors that influence the preferred mechanism of cellular uptake of CPP/cargo complexes.

2. Materials and Methods

2.1. Plasmid construction and protein preparation

We constructed a pR9 plasmid containing a hexa-histidine (6His) and an R9 sequence under the control of the T7 promoter, and a pR9-GFP plasmid containing an additional coding region of *GFP* as previously described.[21] A pQE8-GFP plasmid containing the coding sequence of GFP under the control

of the T5 promoter was kindly provided by Dr. Michael B. Elowitz (Rockefeller University, NY, USA).[21] The pHBT-sGFP(S65T)-NOS plasmid (GenBank Accession No. EF090408) containing an engineered *GFP* gene under the control of the 35S cauliflower mosaic virus enhancer fused to the basal promoter of the maize C4PPDK gene was kindly provided by Dr. Jen Sheen (Harvard University, MA, USA).[25] Plasmid DNA was purified using a Nucleobond AX100 Kit (Machery-Nagel, Duren, Germany). All constructions were verified by DNA sequencing.

For protein expression, pR9, pR9-GFP, and pQE8-GFP plasmids were transformed into *Escherichia coli* and induced as previously described.[39] Expressed proteins were then purified, concentrated and quantified using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Both synthetic nona-arginine (SR9) and histidine-modified nona-arginine (HR9) peptides (MDBio, Taipei, Taiwan) with more than 95% purity were described previously.[24,35] Quantum dots (QDs) with green fluorescent emission at 525 nm were composed of CdSe/ZnS core-shell and carboxyl-functionalized groups on surface (eFluor 525NC, eBioscience, San Diego, CA, USA). A series of concentrations of SR9 or HR9 were premixed with QDs and then incubated at 37°C for 2 h. The optimal molecular ratio of 60 was used in subsequent experiments.

2.2. Cell culture

Human A549 lung cancer cells (American Type Culture Collection, Manassas, VA, USA; CCL-185) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (BioWest, Nuaille, France) supplemented with 10% heat inactivated (56°C for 30 min) bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA). Cells were cultured in a humidified 5% CO_2 and 95% air incubator at 37°C as previously described.[26]

For plant sample, root-tip cells of mung bean (*Vigna radiata* L.) were prepared as previously described.[25]

For cyanobacteria (blue-green algae) culture, both *Synechocystis sp*. PCC 6803 (*American Type Culture Collection*, 27184) and *Synechococcus elongatus* PCC 7942 (ATCC, 33912) (kindly provided by Dr. Yuh-Jang Shieh, Academia Sinica, Taipei, Taiwan) were grown in BG-11 medium with mild shaking at 50 rpm and regular illumination at 28°C.[28]

2.3. Protein transduction and treatment of endocytic modulators

For covalent protein transduction, A549 cells were treated with phosphate buffered saline (PBS), GFP, or R9-GFP for 30 min at 37°C, followed by treatment of Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) as previously described.[35] In the investigation of noncovalent protein transduction, R9 or SR9 peptide was mixed with GFP at a molecular ratio of 3:1 at room temperature for 10 min. All kinds of cells and organisms treated with R9/GFP noncovalent mixtures were described previously.[25,26,28] SR9 peptide was mixed

with plasmid DNA at a nitrogen/phosphate (N/P) ratio of 3 for 30 min, then transferred to another eppendorf tube and incubated with plant cells. After incubation for 30 min, SR9/DNA mixtures were removed by washing with double deionized water.[25] Plant cells were placed on slides after 48 h of treatment and observed under the microscope. In protein transduction tests, 100 nM QDs were premixed with 6 μM SR9 and HR9, respectively for 2 h with shaking as previous described.[35] Then, these complexes were incubated with cells at 37°C CO_2 incubator for 30 min.

A suite of endocytic modulators and physical procedures were used to study mechanisms of internalization. For energy-dependent experiments at 4°C, the protocol of protein transduction was the same as above except that all incubations were performed at 4°C. Cells were preincubated at 4°C for 30 min before being incubated with the protein transduction solution. For endocytic modulator assays, cells were treated with either R9/GFP mixtures, R9/DNA mixtures, SR9/QD mixtures, or HR9/QD mixtures in the absence or presence of 1 mM of *N*-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO, USA), 2 μM of valinomycin (Sigma-Aldrich), 2 μM of nigericin (Fluka Chemie, Seelze, Germany), or 10 mM of sodium azide (Fluka Chemie), respectively. For macropinocytosis and cytoskeleton motions, cells were treated in the absence or presence of 100 μM of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA; Sigma-Aldrich), 10 μM of cytochalasin D (CytD; Sigma-Aldrich) or 10 μM of nocodazole (Sigma-Aldrich), respectively. To deplete or sequester cholesterol from plasma membrane, 5 μg/mL of filipin (Sigma-Aldrich) was added in the culture.[21,24–30,32,35]

2.4. Confocal microscopy

Images were observed using an inverted TMS microscope (Nikon, Melville, NY, USA) equipped with a MD130 CMOS sensor (Electronic Eyepiece, Dar-An, Taiwan) or an Eclipse E600 microscope (Nikon) and recorded using a Penguin 150CL cooled CCD camera (Pixera, Los Gatos, CA, USA). Bright-field, GFP, and blue fluorescent protein (BFP) images were recorded using a BD Pathway 435 System (BD Biosciences, Franklin Lakes, NJ, USA) as previously described.[35] Excitation filters were set at 377/50 and 482/35 nm for blue and green fluorescence, respectively. Emission filters were set at 435LP (long-pass) and 536/40 nm for BFP and GFP channels, respectively. Transmitted light without the excitation filter, but with 536/40 nm emission filter, was used to observe cell morphology as bright-field images. Fluorescent images were acquired by the TCS SL confocal microscope system (Leica, Wetzlar, Germany), and relative intensities of fluorescent images were quantified by the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA) as previously described.[28]

2.5. Flow cytometric analysis

Cells in the control and the experimental groups were harvested and analyzed using a Cytomics FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA,

USA) as previously described.[28] In brief, FL1 filter (excitation 488 nm and emission 515–545 nm) was used for GFP detection. Samples were counted and analyzed by the CXP software.

2.6. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Mean values and SDs were calculated from at least three independent experiments carried out in triplicates per treated group. Statistical comparisons between the control and treated groups were performed by the Student's *t*-test. The levels of statistical significance were set at $P < 0.05$ (*) and $P < 0.01$ (**).

3. Results

3.1. The mechanism of transmembrane delivery of protein by R9

To assess whether arginine-rich CPPs can deliver protein into live cells, human A549 cells were treated with PBS as a control, or R9-GFP fusion protein without fixation followed by treatment with Hoechst 33342. No green signal was observed in the cells treated with PBS as a negative control (Fig. 1) or GFP alone (data not shown) using a BD Pathway 435 System. In contrast, green fluorescence was visible in the cells treated with R9-GFP. Merged images revealed R9-GFP association with the nucleus. These results indicate that R9 peptide mediates the translocation of GFP cargo protein.

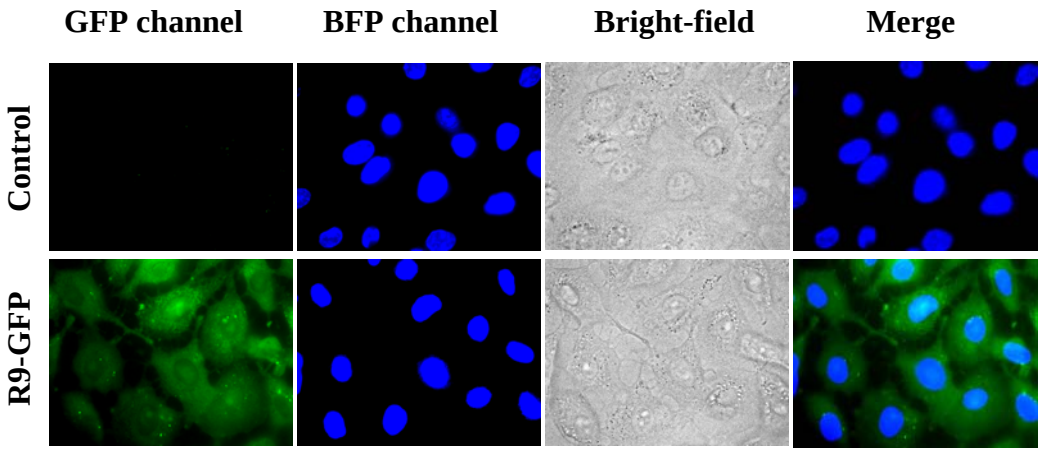

Fig. 1. Cellular entry of R9-GFP into A549 cells. Cells were treated with PBS (control) or 30 μM of R9-GFP for 30 min at 37°C, followed by treatment of Hoechst 33342, as previously described.[35] GFP fluorescence indicates the location of CPP, while the BFP signal shows the location of the nucleus. Images of bright-field, GFP, and BFP channels are shown using a BD Pathway 435 system. Overlap between peptide/protein and nuclei exhibits cyan color in the merged GFP and BFP images.

To compare the mechanism of protein transduction in different species, live human A549 cells, root-tip cells of mung bean, and both 6803 and 7942 strains of cyanobacteria, representing mammalian cells, plant tissues, and prokaryotes, respectively, were used. Macroscopically, the major difference among these three kinds of sample species is that both plant and cyanobacteria contained cell walls. Cyanobacteria, but not plants, have peptidoglycan layers, which define these organisms as Gram-negative bacteria.[40] In our experiments, cells were treated with noncovalent R9/GFP complexes. R9 can deliver GFP into all kinds of cells (data not shown). In mechanistic studies, cells were treated with two macropinocytic inhibitors, EIPA and CytD, for 30 min before exposure to R9/GFP. Both EIPA and CytD reduced protein transduction efficiencies in all cells tested (Fig. 2). These results indicate that macropinocytosis is a route for R9/GFP internalization. Further, A549 cells and root-tip cells of mung bean were very sensitive to EIPA (Fig. 2).

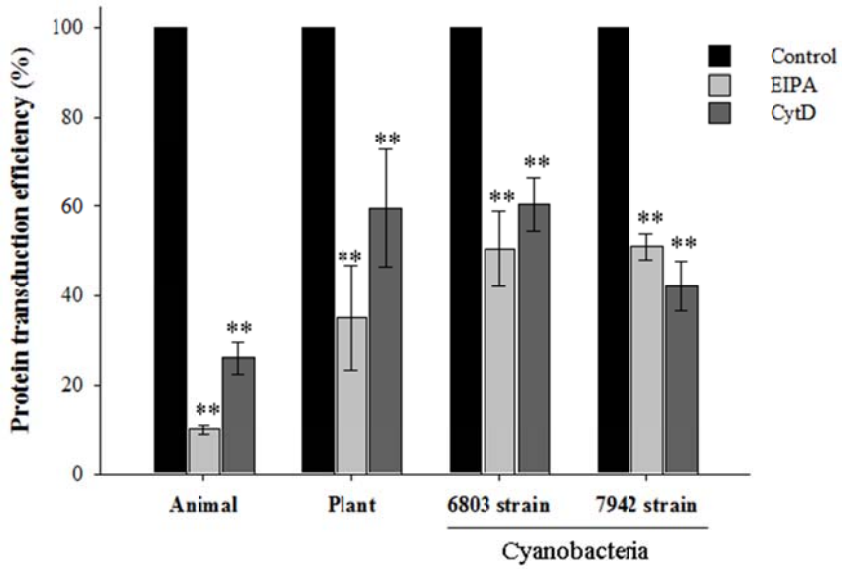

Fig. 2. The Mechanism of cellular uptake of protein by R9 in different cells of species. A549 and mung bean cells were treated with R9/GFP mixtures for 20 min at 37°C. For mechanistic study, cells were pretreated in the absence (control) or presence of 100 μM of EIPA or 10 μM of CytD for 30 min at 37°C, followed by treatment of R9/GFP mixtures for 20 min. Two cyanobacterial strains, PCC 6803 and 7942, were treated with 1 mM of NEM and R9/GFP mixtures in the absence (control in NEM + R9/GFP mixtures) or presence of 100 μM of EIPA or 10 μM of CytD at room temperature. Images were recorded using a confocal microscope, and relative fluorescent intensities of the transported GFP were expressed as mean ± SD. Asterisks indicate statistical significance (* for $p < 0.05$ and ** for $p < 0.01$).

3.2. Mechanisms of transmembrane delivery of QD by different CPPs

To understand the effect of different CPPs on cellular internalization, A549 cells were treated with two synthetic arginine-rich CPPs, SR9 or HR9 peptide. The peptides was premixed with quantum dots (QDs) at the molecular ratio of 60 and incubated at 37°C for 2 h. SR9 and HR9 facilitated the entry of QDs after cells were treated with CPP/QD complexes for 30 min at 37°C.[35] To determine the uptake mechanism, endocytic modulators and physical inhibitors of endocytosis were used. We found that neither chemical nor physical modulators of endocytosis inhibited the HR9-mediated delivery of QDs into A549 cells by flow cytometry (Fig. 3).[34] In the SR9/QD group, reduced uptake was detected in the cells treated with 4°C and EIPA (Fig. 3).[34] Nocodazole and filipin did not decrease uptake in SR9/QD groups (Fig. 3).[34] These results suggested that the mechanism of membrane penetration is CPP-dependent. These data agreed with previous conclusions that SR9/QD enters mammalian cells via multiple pathways,[34] and uptake of HR9/QD is mediated by the direct membrane translocation.[35]

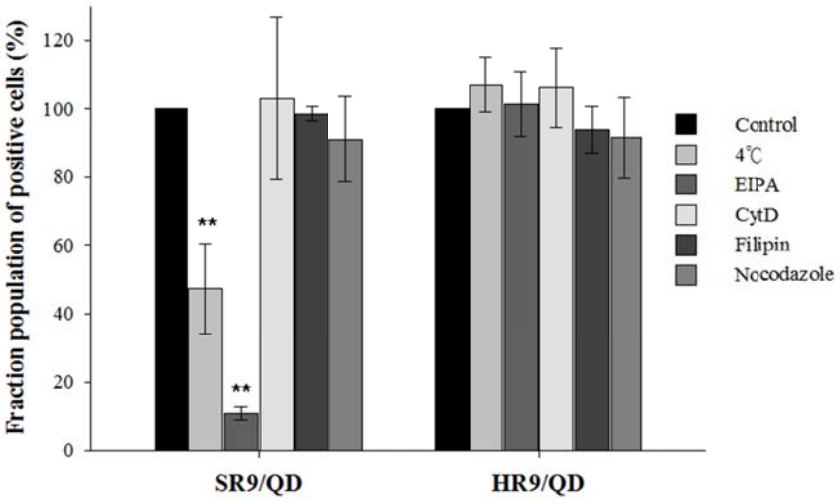

Fig. 3. Effects of different CPPs on cellular uptake of cargo QDs. Cells were incubated with either SR9/QD or HR9/QD complexes in the absence or presence of chemical and physical (4°C) inhibitors of endocytosis at 37°C for 30 min. One hundred μM of EIPA (macropinocytosis inhibitor), 10 μM of CytD (macropinocytosis inhibitor), 5 μg/mL of filipin (lipid-raft inhibitor), and 10 μM of nocodazole (inhibitor of microtubule polymerization) served as pharmacological inhibitors of various endocytic pathways. Transduction efficiency compared to control uptake was analyzed by flow cytometry. Significant differences were set at $P < 0.05$ (*) and $P < 0.01$ (**). The values represented the mean ± SD, n=5.

3.3. Mechanisms of transmembrane delivery of protein and DNA by SR9 in plant

In order to understand the effects of cargo on the mechanism of CPP entry, protein and DNA were mixed with SR9, respectively. Physical and pharmacological inhibitors of endocytosis were chosen to reveal the mechanism of protein transduction in plant. Four °C block all kinds of endocytosis by ATP depletion. NEM, valinomycin, nigericin, and sodium azide were drugs that interfere with various biological processes to stall energy-dependent endocytosis.[32] EIPA is a specific macropinocytic inhibitor whereas CytD obstructs both energy-dependent endocytosis and macropinocytosis.[32] GFP was mixed with SR9 at the molecular ratio of 3:1 and DNA was mixed with SR9 at the optimal N/P ratio of 3:1. Root-tip cells of mung bean were pretreated with different inhibitors for 30 min, and then soaked in SR9/cargo mixtures in the absence or presence of an inhibitor. Our data indicated that cells treated with EIPA and CytD exhibited significant reduction in penetration of SR9/GFP, an indication of macropinocytosis (Fig. 4). Interestingly, the SR9-mediated DNA delivery was interfered with the treatment of 4°C, NEM, and sodium azide, but not valinomycin and nigericin (Fig. 4). Though the role of energy in membrane transduction of GFP protein in plants is still unclear, macropinocytosis inhibitors could reduce the transduction with noticeable differences from DNA transduction. Collectively, we have demonstrated that types of cargoes could play a key role in ways of CPP/cargoes translocation.

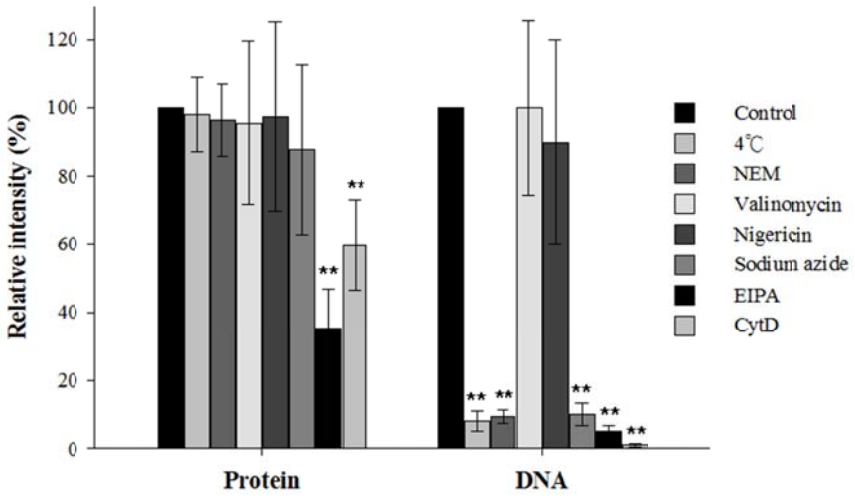

Fig. 4. Mechanisms of cellular uptake of proteins and DNAs mediated by SR9. SR9 peptide was mixed with GFP at the molecular ratio of 3:1 or the pHBT-sGFP(S65T)-NOS plasmid at an N/P ratio of 3:1. Root-tip cells of mung bean were treated with the SR9/cargo mixtures in the absence (control) or presence of 100 μM of EIPA, 10 μM of CytD, 10 mM of sodium azide, 1 mM of NEM, 2 μM of valinomycin or 2 μM of nigericin for 30 min at room temperature. Samples were also treated with the same mixtures at 4°C. Images were recorded using a confocal microscope, and relative intensities of fluorescence, reflecting cellular uptake, were analyzed and quantified using UN-SCAN-IT software. Data were presented as mean ± SD. Significant differences were shown for $P < 0.05$ (*) and $P < 0.01$ (**).

4. Discussion

In pharmacology, endosome forming disrupting drugs (valinomycin and nigericin), a metabolism depression drug (sodium azide), and generally endocytic inhibitors (NEM and 4°C) were thought to interfere with the classical endocytosis.[41–43] Our data demonstrate that arginine-rich CPP/cargo complexes enter cells through macropinocytosis, direct membrane translocation, or multiple endocytic pathways (Fig. 2–4). Direct membrane translocation is an energy-independent pathway, including pore formation, inverted micelle formation, carpet-like perturbation, and membrane thinning model.[44,45] It is generally observed only at high CPP concentrations or with primary amphipathic CPPs.[46] However, we reason that HR9 penetrate cells by pore formation because of the *imidazole* functional *group* on its histidine residues.[35]

Endocytosis is classified into two categories: phagocytosis that involves the uptake of large particles and pinocytosis that involves solute uptake.[44] Macropinocytosis, clathrin-dependent, caveolin-dependent, and clathrin/caveolin-independent pathways are forms of pinocytosis.[47] Many factors affect the route and efficiency of cellular uptake. With CPPs, number of positive charges, conformation, length of the peptide, hydrophobicities, and concentration influence the uptake.[44] Recently, both arginine length of CPP and hydrophobicity of cargo were proven to be important factors for direct membrane translocation.[48,49] In CPP-mediated co-transport, the nature of cargo, cargo size, and method of cargo/CPP association determine the preferred translocation mechanism.[44,48] Our studies also provided the evidence that factors, such as cell types, temperature, and incubation time, influence the uptake mechanism.[44,48–50]

Arginine-rich CPPs are very attractive, nontoxic candidates for the delivery of bio-imaging and therapeutic macromolecules, such as QDs, proteins, and nucleic acids.[20–38] Their safety has been confirmed by metabolic analysis.[51] In the present study, we have used pharmacological modulators and physical treatments to demonstrate that arginine-rich CPP/cargoes can be internalized via macropinocytosis, direct membrane translocation, or a combination of multiple pathways. The specific route of internalization is influenced by the nature of the CPP, the nature of the cargo, and the nature of the CPP/cargo association.

5. Conclusion

In this study, we demonstrated that types of CPPs and cargoes are two primary factors in determining the mechanisms of transmembrane delivery. All of mammalian cells, plant cells, and cyanobacteria were sensitive with varying degrees to macropinocytosis inhibitors EIPA and CytD, indicating that

macropinocytosis is a route for R9/GFP internalization. Our data suggested that different arginine-rich CPPs (SR9 and HR9) carrying the same cargoes (QDs) induce different responses of membrane action. While SR9/QD entered mammalian cells via multiple pathways, the uptake of HR9/QD was a direct membrane translocation in an energy-independent manner. In the case of the same CPPs, R9/GFP crossed membranes by macropinocytosis while the cellular uptake mechanism of R9/DNA was a combination of multiple pathways.

Acknowledgements.

We thank Dr. Michael B. Elowitz (California Institute of technology, CA, USA) for provision of the pQE8-GFP plasmid, Dr. Jen Sheen (Harvard University, MA, USA) for the pHBT-sGFP(S65T)-NOS plasmid, and Dr. Yuh-Jang Shieh (Academia Sinica, Taipei, Taiwan) for cyanobacteria. We are grateful to Dr. Robert S. Aronstam (Missouri University of Science and Technology, USA) for editing the manuscript. This work was supported by Award Number R15EB009530 from the National Institutes of Health (Y.-W.H.) and the National Science Council, Taiwan (H.-J.L.).

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Received: August, 2012