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Chapter 8

MACROPINOCYTOSIS: POSSIBLE MECHANISMS OF CELLULAR ENTRY OF ARGININE-RICH INTRACELLULAR DELIVERY PEPTIDES

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ABSTRACT

Endocytosis, which plays a key role in many different species, is the process that cells take up extracellular materials through plasma membranes. Protein transduction domains (PTDs), also called cell-penetrating peptides (CPPs), are small peptides and contain a large amount of basic amino acids. Several PTDs, including arginine-rich intracellular delivery (AID) peptides, were found to be responsible for cellular uptake of macromolecules. In our previous studies, AID peptides have been proven to either covalently transport proteins or noncovalently internalize proteins, DNAs or RNAs into animal or plant cells. The mechanisms by which PTD enter cells are still in vigorous debate. Our studies indicated that the possible mechanisms of AID peptide-mediated cellular entry might involve a combination of multiple internalization pathways, including at least macropinocytosis. Furthermore, our recent reports demonstrated for the first time that AID peptides could rapidly and efficiently deliver proteins into animal and plant cells in both covalent and noncovalent protein transductions (CNPT) synchronously. Therefore, investigations of cellular uptake mediated by AID peptides

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facilitate our understanding of endocytosis in more details and reveal nonclassically endocytic pathways.

INTRODUCTION

Endocytosis is a process that internalizes extracellular material through plasma membranes. This process is obligate for survival as cells apply this tactic to either obtain essential nutrition or to excrete cellular waste. Endocytosis involves formation of vesicles which later fuse with endosomes, and as such, contents from endocytosis usually enter the endo-lysosomal system in the late period of the process [1]. An endocytic pathway involves many basic cellular mechanisms, such as phospholipid recycling of plasma membranes, protein trafficking, cellular polarity maintenance, down-regulating receptors on cell membrane and antigen presentation [1, 2]. Although endocytosis is crucial to cellular survival, it can also be exploited by pathogens and toxins to allow their entry into cells. Thus, voluminous investigations have been launched to study 1) processes and cellular components that are involved in endocytosis and 2) endocytic roles in physiology and pathology. In the late 1990s, endocytosis was classified into clathrin-dependent and clathrin-independent pathways after the discovery of a caveolae-dependent pathway [3, 4]. Most uptake studies on microorganisms and viruses investigated clathrin-dependent and clathrin-independent pathways. To date, studies have proved to be inconclusive in identifying the number of and the kind of cellular components involving clathrin-independent and caveolae-independent endocytic pathways [3]. According to analysis of the ultrastructure of membrane-transport intermediates, endocytic pathways were further classified into four categories: clathrin-dependent, caveolae-dependent, polymorphous tubes formation and macropinocytosis [3].

Tat protein derived from human immunodeficiency virus type 1, discovered in 1988, was thought to have potential in overcoming the barrier of plasma membrane during cellular internalization [5, 6]. Protein transduction domain (PTD), also named as cell-penetrating peptide (CPP), is the functional domain in Tat protein which gives rise to its membrane penetrating ability [7]. Within the domain, it contains a large amount of basic amino acids which are important to its efficiency of protein transduction [8]. Not only can PTD translocate into cells by itself, but it also can transport other cargoes into cells together. These cargoes carried by PTDs vary widely in types, such as proteins, nucleic acids, peptide nucleic acids, nanoparticles and liposomes. The size of these cargoes can be even larger than 200 nm in diameter [9, 10]. In our previous studies, we utilized PTD analogues called arginine-rich intracellular delivery (AID) peptides (i.e., nona-arginine) and demonstrated that AID peptides could deliver biologically active macromolecules into different kinds of cells in both covalent and noncovalent manners [11–17]. The kinetics of cellular entry of PTD peptides were measured at a half-time of 1.8 min, corresponding to a first-order rate constant of 0.007 sec^{-1} at a peptide concentration of 100 μM [18].

The mechanism of PTDs internalization is still in vigorous debate. A previous study indicated that internalization of PTDs is energy-independent [8]; therefore, low temperature could not prohibit cellular entry of PTDs. Kawamura *et al*. demonstrated that p53-Tat fusion protein enters CHO cells via a clathrin-dependent endocytosis [19]. Other investigations supported the notion that PTDs cross-linked with cargoes utilize a caveolae-dependent pathway for internalization in various cells [20–22]. In addition to caveolin-mediated

pathway, recent studies have focused on another major but nonclassical pathway, macropinocytosis, in cellular internalization of PTDs alone or conjugated with cargoes covalently [23–26]. Our group is a pioneer in the study of plant species on transduction mechanisms of AID peptides covalently conjugated with fluorescent protein (FP). Our data indicated that internalization of FP-AID peptides is neither clathrin-dependent nor caveolae-dependent [11]. While we changed the FP and AID peptides linkage from covalent to noncovalent manner, their entry into cells was both receptor- and energy-independent. Further investigation indicated that macropinocytosis was the mechanism of action in delivering AID peptides in various types of cells [13, 15, 17]. Our most recent work demonstrated that AID peptides could enter cells in both covalent and noncovalent protein transductions (CNPT), and that AID peptides could deliver two kinds of FP into cells in covalent and noncovalent fashions synchronously [27].

In this report, we summarize uptake efficiency and mechanistic studies on covalent, noncovalent and combined transduction of various types of cargoes in plant species, mammalian species and cyanobacterial strains. We start this article by describing protocols and procedures of transduction. Particularly, detailed attention focuses on construction and preparation of plasmids from our laboratory, optimization, induction and purification of recombinant proteins, as well as treatment of cells and use of endocytic modulators aimed at various mechanisms of transduction. Data are compared and contrasted between covalent protein transduction (CPT), noncovalent protein transduction (NPT), and CNPT. At the end, we summarize others' and our discovery and point out research for the future.

MATERIALS AND METHODS

Plasmid Construction and Protein Preparation

The pR9 plasmid we constructed contains a hexa-histidine (6His) and a nona-arginine (R9) sequence under the control of the T7 promoter, while the pR9-GFP plasmid we made has an additional coding region of green fluorescent protein (GFP) inserted into the pR9 vector as previously described [11]. The pQE8-GFP plasmid (kindly provided by Dr. Michael B. Elowitz, Rockefeller University, NY, USA) consists of a coding sequence of GFP under the control of the T5 promoter [11]. The mCherry plasmid (kindly provided by Dr. Roger Y. Tsien, University of California, San Diego, CA, USA) has a coding sequence of 6His-tagged red fluorescent protein under the control of the T7 promoter [12]. The pHBT-sGFP(S65T)-NOS plasmid (GenBank Accession No. EF090408, kindly provided by Dr. Jen Sheen, Harvard University, MA, USA) contains an engineered *gfp* gene under the control of the 35S cauliflower mosaic virus enhancer fused to the basal promoter of the maize C4PPDK gene [15]. Plasmid DNA was purified with the Nucleobond AX100 Kit (Machery-Nagel, Duren, Germany). All constructions were verified by DNA sequencing.

For protein expression, pR9, pR9-GFP, pQE8-GFP and mCherry plasmids were transformed into *Escherichia coli* and induced as previously described [13, 27]. Expressed proteins were then purified, concentrated and quantified by the Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The chemically synthetic nona-arginine (SR9) peptide (MDBio, Taipei, Taiwan) with more than 95% purity was described previously [13].

Preparation of Various Cells

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 [14, 27].

Onion (*Allium cepa* L.) bulbs, root tips of mung bean (*Vigna radiata* L.), soybean (*Glycine max* L.) and corn (*Zea mays* L.) were prepared as previously described [11, 13, 15]. For cyanobacteria (blue-green algae) culture, both *Synechocystis sp*. PCC 6803 (ATCC, 27184) and *Synechococcus elongatus* PCC 7942 (ATCC, 33912) (kindly provided by Dr. Yuh-Jang Shieh, National Defense Medical Center, Taiwan) were grown in BG-11 medium with mild shaking at 50 rpm and regular illumination at 28°C [17].

Protein Transduction and Treatment of Endocytic Modulators

In the study on CPT, plant cells were mixed with 2.7 μM of R9-GFP covalent fusion protein for different periods of time and washed with distilled water to remove free protein. In the investigation of NPT, 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 tissues treated with AID peptides/FP noncovalent mixtures were described previously [11, 13, 14, 17]. SR9 peptide was mixed with plasmid DNA at the nitrogen/phosphate (N/P) ratio of 3 for 30 min, then transferred to another eppendorf tube and incubated with plant tissues. After incubation for 30 min, SR9/DNA mixtures were removed by washing with double deionized water [15]. Plant tissues were placed on slides after 48 h of treatment and observed under the microscope. For CNPT test, A549 cells were treated with 10 μM of mCherry premixed with 30 μM of R9-GFP at the molecular ratio (R9-GFP:mCherry) of 3:1 for 10 min at 37°C and washed with phosphate buffered saline [27].

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, AID peptides/FP mixtures, AID peptides/DNA mixtures or R9-GFP/FP mixtures in the absence or presence of 1 mM of *N*-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO, USA), 1.5 μM of okadaic acid (Sigma-Aldrich), 2 μM of valinomycin (Sigma-Aldrich), 2 μM of nigericin (Fluka Chemie, Seelze, Germany), 10 mM of sodium azide (Fluka Chemie) or 80 mM of sodium chlorate ($NaClO_3$; Sigma-Aldrich), 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 and impair lipid raft formation, 2 mM of methyl-β-cyclodextrin (MβCD; Sigma-Aldrich) were added in the culture [11, 13–15, 17, 27].

Confocal Microscopy

Images were observed under an inverted TMS microscope (Nikon, Melville, NY, USA) equipped with a MD130 CMOS sensor (Electronic Eyepiece, Dar-An, Taiwan) or under an Eclipse E600 microscope (Nikon) and recorded by a Penguin 150CL cooled CCD camera (Pixera, Los Gatos, CA, USA). 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 [17].

Statistical Analysis

Results were expressed as means ± standard deviations (SDs). Statistical comparisons between the control and treated groups were performed by Student's *t*-test. The levels of statistical significance were set at $P < 0.05$ (*).

RESULTS

Mechanisms of Covalent Protein Transduction (CPT) for Protein Delivery

In order to reveal mechanisms of cellular internalization of AID peptides in a covalent manner, plant cells were treated with R9-GFP fusion protein at different time points in the presence or absence of various inhibitors. R9-GFP was internalized within 5 min in cells (Figure 1, control), while other controls were described previously [11]. The fluorescence emitted by internalized protein could remain in plant cells stably more than 2 days after protein transduction. Low temperature (4°C) incubation and treatment of endocytic inhibitors (NEM and okadaic acid) did not alter AID peptide internalization in CPT manner (Figure 1). These results suggested that cellular internalization of R9-GFP was independent of energy and classical endocytosis.

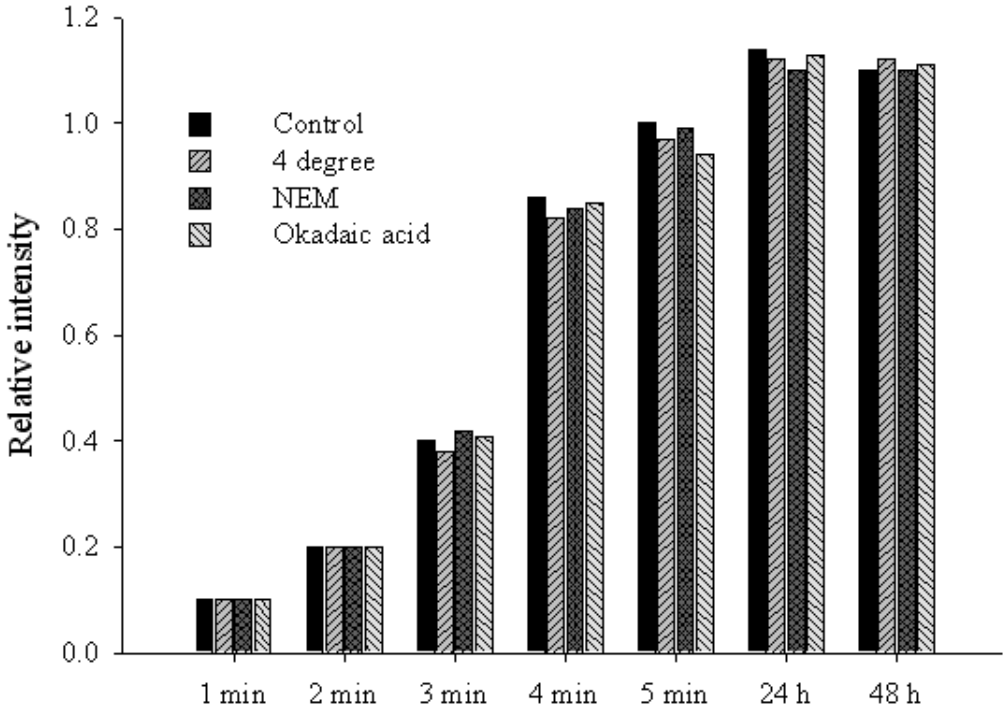
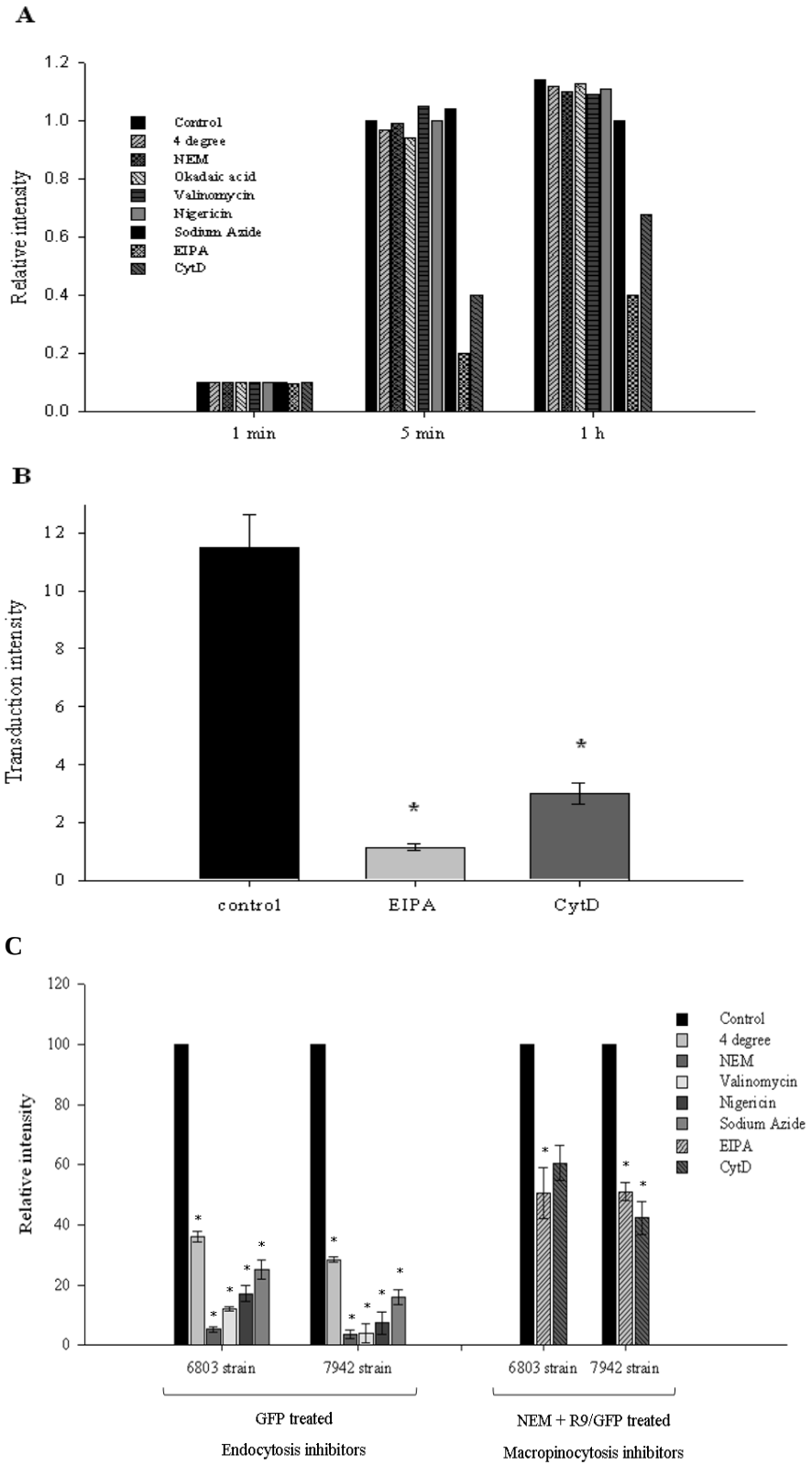


Figure 1. Mechanisms of covalent protein transduction (CPT) for protein delivery. Onion epidermal cells were treated with R9-GFP in the absence (control) or presence of 1 mM of NEM or 1.5 μM of okadaic acid at room temperatures. Cells were also treated with the same protein solution at 4°C. Relative fluorescent intensities (y-axis) at different times (x-axis; 1, 2, 3, 4, 5 min, 24 h and 48 h) after protein internalization were analyzed with the UN-SCAN-IT software.

Mechanisms of Noncovalent Protein Transduction (NPT) for Protein Delivery

For cellular internalization of AID peptides in a noncovalent fashion, cells from different species were treated with AID peptides/GFP mixtures. In the absence of drugs or physical treatment, protein internalization in plant cells was detected at 5 min of incubation with R9/GFP mixtures (Figure 2A, control). Similar to the results in CPT in plants, low temperature and endocytic modulators, such as NEM, okadaic acid, valinomycin, nigericin and sodium azide did not interfere AID peptide internalization in NPT (Figure 2A). These results suggested that either energy-dependent or classical endocytosis pathway was not the major pathway in NPT. Contrarily, treatment of plant cells with macropinocytosis inhibitors EIPA and CytD resulted in a dramatic reduction in cellular entry indicating that NPT in plant cells involve macropinocytosis and actin rearrangement.


A
Relative intensity
1.2
1.0
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Control
4 degree
NEM
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Valinomycin
Nigericin
Sodium Azide
EIPA
CytD
1 min
5 min
1 h
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Transduction intensity
12
10
8
6
4
2
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control
EIPA
CytD
C
Relative intensity
120
100
80
60
40
20
0
Control
4 degree
NEM
Valinomycin
Nigericin
Sodium Azide
EIPA
CytD
6803 strain
7942 strain
6803 strain
7942 strain
GFP treated
Endocytosis inhibitors
NEM + R9/GFP treated
Macropinocytosis inhibitors

Figure 2. Mechanisms of noncovalent protein transduction (NPT) for protein delivery in different species. (A). The mechanism of NPT in plant cells. Onion epidermal cells were treated with AID peptides/GFP mixtures in the absence (control) or presence of 1 mM of NEM, 1.5 μM of okadaic acid, 2 μM of valinomycin, 2 μM of nigericin, 10 mM of sodium azide, 100 μM of EIPA or 10 μM of CytD at different temperatures (room temperature or 4°C). Relative intensities at different time (1 min, 5 min and 1 h) were analyzed with the UN-SCAN-IT software. (B). The mechanism of NPT in mammalian cells. A549 cells were treated with SR9/GFP mixtures for 20 min at 37°C in the absence (control) or presence of EIPA or CytD at the same concentrations as described above. Relative intensities in different treatment were represented by mean ± SD. Significant differences were presented as an asterisk (*, $p < 0.05$). (C). The mechanism of NPT in cyanobacteria. Two cyanobacterial strains, PCC 6803 and 7942, were incubated with GFP only in the absence (control in GFP treated group) or presence of endocytic modulators (1 mM of NEM, 2 μM of valinomycin, 2 μM of nigericin, or 10 mM of sodium azide) in either room temperature or 4°C. Cyanobacteria were treated with NEM and AID peptides/GFP mixtures in the absence (control in NEM + SR9/GFP mixtures) or presence of 100 μM of EIPA or 10 μM of CytD at room temperature. An asterisk indicates significant differences ($p < 0.05$) between the control and the experimental group.

Similar results of NPT were observed in mammalian cells (Figure 2B). Human A549 lung cancer cells treated with AID peptides/GFP mixtures for 20 min at 37°C in the presence of EIPA or CytD dramatically decreased NPT.

Interestingly, cyanobacteria displayed the ability of GFP uptake without facilitation by AID peptides (Figure 2C). Therefore, in order to test the mechanism of NPT in AID peptides/GFP mixtures, we chose various endocytic modulators to inhibit the GFP uptake in cyanobacteria. Both energy depletion and classical endocytic modulators successfully prevented the GFP uptake with NEM having the highest prohibitive capacity (Figure 2C). Cells were treated with NEM and R9/GFP mixtures in the absence or presence of EIPA or CytD. Both PCC 6803 and 7942 strains did not internalize R9/GFP in the presence of EIPA. Uptake inhibition by CytD was only observed in PCC 7942 strain. These data suggested that AID peptide-dependent NPT in cyanobacteria was mediated by macropinocytosis.

Mechanisms of Noncovalent Protein Transduction (NPT) for DNA Delivery

To understand the mechanism of AID peptide-dependent DNA entry into plant cells in NPT, plant cells were treated with SR9/pHBT-sGFP(S65T)-NOS plasmid mixtures for 30 min in the absence or presence of different kinds of endocytic modulators. After 48 h of incubation in the absence of modulators, the *gfp* reporter gene was expressed and the emission of green fluorescence represented the degree of plasmid DNA translocation via AID peptides in cells (Figure 3, control). Surprisingly, fluorescent intensities in the treatment groups with EIPA, CytD, low temperature, sodium azide or NEM were significantly reduced, while fluorescent intensities in the treatment of valinomycin or nigericin were similar to that of the control (Figure 3). These results indicated that the mechanism of AID peptide-mediated DNA internalization in NPT might differ from those of CPT and NPT for protein delivery.

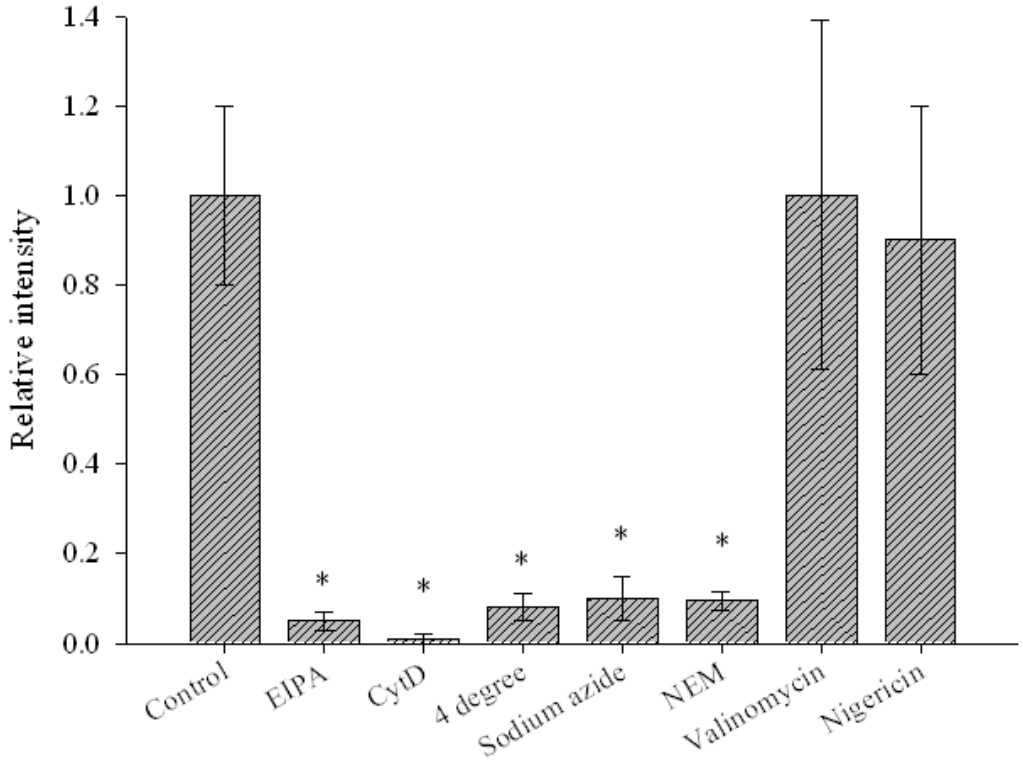

Figure 3. Mechanisms of noncovalent protein transduction (NPT) for DNA delivery. SR9 peptide was mixed with the pHBT-sGFP(S65T)-NOS plasmid at the N/P ratio of 3. Root tips of mung bean were treated with the peptide/plasmid mixtures in the absence (control) or presence of 100 μM of EIPA, 10 μM of CytD, 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. Relative intensities of fluorescence were quantified after 48 h of treatment. An asterisk indicates significant differences ($p < 0.05$).

Mechanisms of Covalent and Noncovalent Protein Transductions (CNPT) for Protein Delivery

To elucidate mechanisms of CNPT for protein delivery, human A549 cells were treated with R9-GFP/mCherry mixtures. We found that green fluorescent intensities in all treatment groups with endocytic modulators were significantly reduced except for the 4°C treatment compared to that of the control (Figure 4, R9-GFP group). Contrarily, red fluorescent intensities decreased in treatment with EIPA, CytD or nocodazole, but remained similar intensities in treatment with low temperature, MβCD or sodium chlorate (Figure 4, mCherry group). Moreover, the highest transduction reduction was the group treated with CytD, an inhibitor of actin rearrangement, which involved many kinds of physiological process (Figure 4). These results demonstrated that mechanisms of CNPT differed from those of CPT and NPT for protein delivery. Potential uptake mechanism of CNPT may involve a combination of multiple internalization pathways.

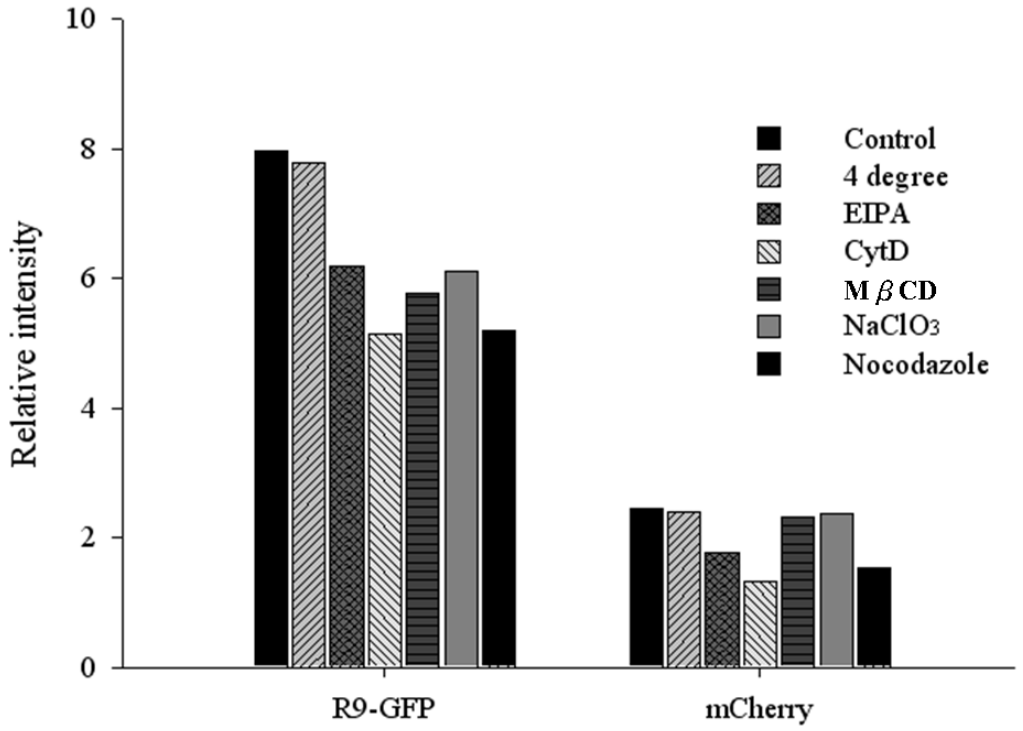

Figure 4. Mechanisms of covalent and noncovalent protein transductions (CNPT) for protein delivery. A549 cells were treated with R9-GFP/mCherry mixtures at the molecular ratio of 3:1 in the absence (control) or presence of different modulators (100 μM of EIPA, 10 μM of CytD, 2 mM of MβCD, 80 mM of sodium chlorate or 10 μM of nocodazole) at different temperatures (37°C or 4°C). Green and red fluorescent images were quantified respectively with the UN-SCAN-IT software and were presented as relative intensities (y-axis).

CONCLUSION

In this study, we have demonstrated that AID peptides possess the ability to efficiently deliver different types of cargoes including proteins and DNAs into various species in covalent, noncovalent or combined covalent and noncovalent manners. Not only can these cargoes be translocated into and stay in cells, but they also retain their activities for a certain period of time. The histograms of CPT and NPT showed that R9-GFP and AID peptides/GFP complexes could internalize into cells as fast as 1 min after treatment, and fluorescent intensities reached near maximum after incubation for 5 min (Figure 1A and 2A). Various types of modulators were chosen to study mechanisms of uptake: classical endocytic modulators (low temperature, NEM, valinomycin, nigericin, sodium azide and sodium chlorate), macropinocytosis inhibitors (EIPA and CytD), cytoskeleton motion inhibitors (CytD and nocodazole) and a lipid raft inhibitor (MβCD). Treatment with classical endocytic

inhibitors or energy depletion was unable to prevent AID peptides from internalization by CPT, NPT and CNPT in plant and mammalian cells (Figure 1, 2 and 4). However, EIPA and CytD were effective inhibitors which significantly reduced protein transduction (Figure 2B, 2C, 3 and 4). Collectively our results indicated that macropinocytosis is the major pathway for AID peptides internalization. Yet, it is likely that AID peptide-mediated DNA delivery in NPT and protein transport in CNPT involve a combination of multiple endocytic pathways.

Findings in other reports were consistent with ours in regards to energy-independent CPT, NPT and CNPT. Results from different laboratories indicated that Tat-associated peptides, such as polyarginine or AID peptides, entered cells via an energy-independent manner [7, 8, 10, 25]. Whether internalization of PTDs is mediated by classical endocytosis remained debatable. Recently, endocytic pathways were further divided into ten different types according to their molecular characteristics and implicated cargoes [28]. Endosome membrane potential collapsing drugs (valinomycin and nigericin), a metabolism depression drug (sodium azide) and general endocytic inhibitors (NEM and okadaic acid) were thought to interfere with classical endocytosis [29–31]. However, our data demonstrated that AID peptides were not affected by these drugs in CPT and NPT (Figure 1 and 2). A study showed that PTD entered cells via a clathrin-dependent endocytosis [19], while others proposed caveolae-dependent endocytosis as the major route for PTD internalization [20–22].

Macropinocytosis, first described in 1931, projected distinct structural ruffles on the plasma membrane, and this finding extended the understanding in endocytosis [32, 33]. Macropinocytosis is accompanied by a rapid increase in uptake and extensive leading-edge membrane ruffling that can be observed in 10 min in the experiment [34]. After internalization, inward movement of mature macropinosomes to the perinuclear region involves many cytoskeleton motions, including myosin, dynein and microtubule [35]. Macropinocytosis is sensitive to EIPA and CytD [23, 24]. The EIPA is a Na^+/H^+ exchanged inhibitor which specifically down-regulates ionic equilibrium in macropinocytosis [23, 24, 34]. CytD is an F-actin polymerization inhibitor which disrupts actin dynamics. Our data (Figure 2–4) revealed that AID peptide-mediated internalizations were reduced by treatment with EIPA and CytD. It is worth noting that PTD promoted undefined actin motion and induced the formation of lamellipodia [36, 37]. Together, a lot of endocytic phenomena including classical endocytosis and macropinocytosis were related to actin rearrangement [19, 36].

The size of plasma membrane invaginations may influence routes of internalization. Caveolae-dependent endosomes containing cholesterol, sphingolipids, receptors and caveolins were about 50–80 nm in size [38]. Clathrin-coated pits were around 100–200 nm [39]. Macropinosomes formed from macropinocytosis ranged from 0.2 to 5 μm in diameter [40]. It provided a route for macromolecules to enter cells by a nonselective endocytosis, and large molecules fused or mixed with PTD complexes were likely to be carried out by macropinocytosis. However, size is not the only factor determining the route as molecule charges on the cationic peptides offer strong interactions with heparin sulfate [38]. Therefore, the nature of cargoes plays a determining role in the mechanism of AID peptide-mediated entry as well.

Cell types and condition could also affect on the mechanism of AID peptides uptake [41, 42]. Recent studies suggested that AID peptides entered cells efficiently at 4°C via direct translocation, but cells might uptake AID peptides by macropinocytosis, pinocytosis or direct translocation at the temperature which was higher than 12°C [43]. Factors influencing uptake

include, but not limit to, concentrations of AID peptides alone or molecules combined with AID peptides and environmental temperature. According to these investigations, it was noted that there were a lot of factors, which were able to determine and change the mechanism of AID peptide-mediated cellular uptake. Our studies also provided the evidence to support this proposition (Figure 1–4).

Collectively, we have demonstrated that feasibility of AID peptide-mediated cellular uptake of exogenous substances. AID peptides can enter various types of cells of animals, plants and microorganisms by CPT, NPT and CNPT. Specifically, by using modulators and physical treatment we have demonstrated that AID peptides can be internalized via a combination of multiple pathways. More importantly, routes of internalization are species specific [17]. These investigations shed light on details in endocytosis and provided further applications for the future.

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ABBREVIATIONS

AID	arginine-rich intracellular delivery;
CNPT	covalent and noncovalent protein transductions;
CPP	cell-penetrating peptide;
CPT	covalent protein transduction;
CytD	cytochalasin D;
EIPA	5-(*N*-ethyl-*N*-isopropyl)-amiloride;
FP	fluorescent protein;
GFP	green fluorescent protein;
6His	hexa-histidine;
MβCD	methyl-β-cyclodextrin;
NEM	*N*-ethylmaleimide;
N/P	nitrogen/phosphate;
NPT	noncovalent protein transduction;
PTD	protein transduction domain;
R9	nona-arginine;
SR9	synthetic nona-arginine.

REFERENCES

[1] Smythe, E. & Ayscough, K. R. (2006) Actin regulation in endocytosis. *J. Cell Sci.* 119, 4589–4598.

[2] Mukherjee, S., Ghosh, R. N. & Maxfield, F. R. (1997) Endocytosis. *Physiol. Rev.* 77, 759–803.

[3] Hansen, C. G. & Nichols, B. J. (2009) Molecular mechanisms of clathrin-independent endocytosis. *J. Cell Sci.* 122, 1713–1721.

[4] Parton, R. G. (1996) Caveolae and caveolins. *Curr. Opin. Cell Biol.* 8, 542-548.

[5] Green, M. & Loewenstein, P. M. (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* 55, 1179–1188.

[6] Frankel, A. D. & Pabo, C. O. (1988) Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 55, 1989–1993.

[7] Vives, E., Brodin, P. & Lebleu, B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 272, 16010–16017.

[8] Futaki, S. (2002) Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms. *Int. J. Pharm.* 245, 1–7.

[9] Schwarze, S. R., Ho, A., Vocero-Akbani, A. & Dowdy, S. F. (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 285, 1569–1572.

[10] Wadia, J. S. & Dowdy, S. F. (2002) Protein transduction technology. *Curr. Opin Biotechnol.* 13, 52–56.

[11] Chang, M., Chou, J. C. & Lee, H. J. (2005) Cellular internalization of fluorescent proteins via arginine-rich intracellular delivery peptide in plant cells. *Plant Cell Physiol.* 46, 482–488.

[12] Wang, Y. H., Chen, C. P., Chan, M. H., Chang, M., Hou, Y. W., Chen, H. H., Hsu, H. R., Liu, K. & Lee, H. J. (2006) Arginine-rich intracellular delivery peptides noncovalently transport protein into living cells. *Biochem. Biophys. Res. Commun.* 346, 758–767.

[13] Chang, M., Chou, J. C., Chen, C. P., Liu, B. R. & Lee, H. J. (2007) Noncovalent protein transduction in plant cells by macropinocytosis. *New Phytol.* 174, 46–56.

[14] Hou, Y. W., Chan, M. H., Hsu, H. R., Liu, B. R., Chen, C. P., Chen, H. H. & Lee, H. J. (2007) Transdermal delivery of proteins mediated by non-covalently associated arginine-rich intracellular delivery peptides. *Exp. Dermatol.* 16, 999–1006.

[15] Chen, C. P., Chou, J. C., Liu, B. R., Chang, M. & Lee, H. J. (2007) Transfection and expression of plasmid DNA in plant cells by an arginine-rich intracellular delivery peptide without protoplast preparation. *FEBS Lett.* 581, 1891–1897.

[16] Wang, Y. H., Hou, Y. W. & Lee, H. J. (2007) An intracellular delivery method for siRNA by an arginine-rich peptide. *J. Biochem. Biophys. Methods* 70, 579–586.

[17] Liu, B. R., Chou, J. C. & Lee, H. J. (2008) Cell membrane diversity in noncovalent protein transduction. *J. Membr. Biol.* 222, 1–15.

[18] Ziegler, A., Nervi, P., Durrenberger, M. & Seelig, J. (2005) The cationic cell-penetrating peptide CPP (TAT) derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence. *Biochemistry* 44, 138–148.

[19] Kawamura, K. S., Sung, M., Bolewska-Pedyczak, E. & Gariepy, J. (2006) Probing the impact of valency on the routing of arginine-rich peptides into eukaryotic cells. *Biochemistry* 45, 1116–1127.

[20] Equchi, A., Akuta, T., Okuyama, H., Senda, T., Yokoi, H., Inokuchi, H., Fujita, S., Hayakawa, T., Takeda, K., Hasegawa, M. & Nakanishi, M. (2001) Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells. *J. Biol. Chem.* 276, 26204–26210.

[21] Fervari, A., Pellegrini, V., Arcangeli, C., Fittipaldi, A., Giacca, M. & Beltram, F. (2003) Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Mol. Ther.* 8, 284–294.

[22] Fittipaldi, A., Ferrari, A., Zoppé, M., Arcangeli, C., Pellegrini, V., Beltram, F. & Glacca, M. (2003) Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J. Biol. Chem*. 278, 34141–34149.

[23] Wadia, J. S., Stan, R. V. & Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med*. 10, 310–315.

[24] Kaplan, I. M., Wadia, J. S. & Dowdy, S. F. (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J. Control Release* 102, 247–253.

[25] Nakase, I., Takeuchi, T., Tanaka, G. & Futaki, S. (2008) Methodological and cellular aspects that govern the internalization mechanisms of arginine-rich cell-penetrating peptides. *Adv. Drug Deliv. Rev*. 60, 598–607.

[26] El-Sayed, A., Futaki, S. & Harashima, H. (2009) Delivery of macromolecules using arginine-rich cell-penetrating peptides: ways to overcome endosomal entrapment. *AAPS J*. 11, 13–22.

[27] Hu, J. W., Liu, B. R., Wu, C. Y., Lu, S. W. & Lee, H. J. (2009) Protein transport in human cells mediated by covalently and noncovalently conjugated arginine-rich intracellular delivery peptides. *Peptides* 30, 1669–1678.

[28] Doherty, G. J. & McMahon, H. T. (2009) Mechanisms of endocytosis. *Annu. Rev. Biochem*. 78, 857-902.

[29] Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K. & Sugiura, Y. (2002) Possible existence of common internalization mechanisms among arginine-rich peptides. *J. Biol. Chem.* 277, 2437–2443.

[30] Malecki, J., Wiedlocha, A., Weache, J. & Olsnes, S. (2002) Vesicle transmembrane potential is required for translocation to the cytosol of externally added FGF-1. *EMBO J.* 21, 4480–4490.

[31] Hammond, T. G., Goda, F. O., Navar, G. L., Campbell, W. C., Majewski, R. R., Galvan, D. L., Pontillon, F., Kaysen, J. H., Goodwin, T. J., Paddock, S. W. & Verroust, P. J. (1998) Membrane potential mediates H^+-ATPase dependence of degradative pathway endosomal fusion. *J. Membr. Biol.* 162, 157–167.

[32] Lewis, W. H. (1931) Pinocytosis. *Bull. Johns Hopkins Hosp.* 49, 17–23.

[33] Hacker, U., Albrecht, R. & Maniak, M. (1997) Fluid-phase uptake by macropinocytosis in Dictyostelium. *J. Cell Sci.* 110, 105–112.

[34] Meier, O., Boucke, K., Hammer, S. V., Keller, S., Stidwill, R. P., Hemmi, S. & Greber, U. F. (2002) Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J. Cell Biol.* 158, 1119–1131.

[35] Tacon, D., Knight, P. J. & Peckham, M. (2004) Imaging myosin 10 in cells. *Biochem. Soc. Trans.* 32, 689–693.

[36] Nakase, I., Niwa, M., Takeuchi, T.; Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S., Ueda, K., Simpson, J. C., Jones, A. T., Sugiura, Y. & Futaki, S. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol. Ther*. 10, 1011–1022.

[37] Nakase, I., Tadokoro, A., Kawabata, N., Takeuchi, T., Katoh, H., Hiramoto, K., Negishi, M., Nomizu, M., Sugiura, Y. & Futaki, S. (2007) Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. *Biochemistry* 46, 492–501.

[38] Lajoie, P. & Nabi, I. R. (2007) Regulation of raft-dependent endocytosis. *J. Cell Mol. Med*. 11, 644–653.

[39] Ehrlich, M., Boll, W., Van Oijen, A., Hariharan, R., Chandran, K., Nibert, M. L. & Kirchhausen, T. (2004) Endocytosis by random initiation and stabilization of clathrin-coated pits. *Cell* 118, 591–605.

[40] Swanson, J. A. & Watts, C. (1995) Macropinocytosis. *Trends. Cell Biol.* 5, 424–428.

[41] Poon, G. M. K. & Gariepy, J. (2007) Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. *Biochem. Soc. Trans*. 35, 788–793.

[42] Xia, T., Kovochich, M., Liong, M., Zink, J. I. & Nel, A. E. (2008) Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways. *ACS Nano*. 2, 85–96.

[43] Fretz, M. M., Penning, N. A., Al-Taei, S., Futaki, S., Takeuchi, T., Nakase, I., Storm, G. & Jones, A. T. (2007) Temperature-, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells. *Biochem. J.* 403, 335–342.