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ORIGINAL ARTICLES

# Cell Membrane Diversity in Noncovalent Protein Transduction

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**Abstract** Crossing of the plasma membrane for all macromolecules without energy, receptors or any artificial methods was thought to be difficult. Our previous studies demonstrated that arginine-rich intracellular delivery (AID) peptides are able to deliver macromolecules, such as proteins, RNAs and DNAs, into either animal or plant cells. Cellular internalization could be mediated by effective and nontoxic AID peptides in either a covalent or noncovalent protein transduction (NPT) manner. AID peptides were so versatile that the procedure seemed to replace the current artificial transfection methods. However, the utilization of AID peptides has been limited to animal or plant systems so far. None has proposed that AID peptides could work in other species. Here, we select some representative organisms to screen whether NPT mediated by AID peptides works in them. They include cyanobacteria, bacteria, archaea, algae, fungi and yeasts. The results reveal that not all living beings possess this capability of protein transduction. Interestingly, all species of prokaryotes tested, which were thought to be highly diverse from the animal and plant systems, appear to be capable of NPT. The mechanism of AID-mediated NPT in cyanobacteria is in a classical endocytosis- and energy-independent pathway and may involve macropinocytosis. In contrast, green algae and multicellular fungi of the eukaryotes are impermeable to protein passage. Our results bring an interesting clue to the reexamination of the phylogeny of both algae and fungi.

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## Introduction

Uptake of macromolecules into cells is never easy without receptors, specific transporters or endocytosis. The plasma membrane of eukaryotic cells, which is lipophilic in nature, is the barrier that prevents the passage of hetero-molecules and is against the imbalance of the osmotic pressure. Protein transduction domains (PTDs), also called "cell penetrating peptides," are small (fewer than 20 amino acids) and basic residue-rich peptides originally identified from the Tat *trans*-activating transcriptional protein of human immunodeficiency virus type 1 (HIV-1) (Frankel & Pabo, 1988; Green & Loewenstein, 1988). When PTD is synthesized in the form of recombinant fusion protein or as a covalent crosslink to a wide variety of cargoes, this cationic peptide can facilitate the uptake of biologically active macromolecules into mammalian cells (Dowdy & Snyder, 2005). Macromolecules carried by PTDs include peptides/proteins, nucleic acids, peptide nucleic acids, liposomes, nanoparticles and others (Dietz & Bahr, 2004). In addition, PTDs can facilitate systemic delivery of fused proteins to various tissues in living mice (Schwarze et al., 1999; Cai et al., 2006).

Covalent protein transduction mediated by an arginine-rich intracellular delivery (AID) peptide, a more effective PTD, in plants was previously demonstrated by our group (Chang, Chou & Lee, 2005a). We found that an AID peptide can efficiently deliver covalently fused fluorescent proteins, such as green fluorescent protein (GFP) and red fluorescent protein (RFP), into several types of plant cells.

Recently, we demonstrated that protein transduction can be effected in a noncovalent fashion, called "noncovalent protein transduction" (NPT) (Wang et al., 2006; Chang et al., 2007; Chen et al., 2007; Hou et al., 2007; Liu et al., 2007; Wang, Hou & Lee, 2007). An AID peptide can efficiently deliver noncovalently mixed protein (Wang et al., 2006; Chang et al., 2007; Hou et al., 2007; Liu et al., 2007), RNA (Wang et al., 2007) or DNA (Chen et al., 2007) into living cells or dermal tissues in fully active forms. The mechanism of protein transduction mediated by PTDs in living cells has been under vigorous debate. Previous studies reported that protein transduction delivering proteins into cells appears to be independent of classical endocytosis, energy, receptors or active transporters (Schwarze, Hruska & Dowdy, 2000; Lindsay, 2002; Wadia & Dowdy, 2002). Recent work proposed that a specialized form of endocytosis, known as "macropinocytosis," plays a major role in the uptake of both PTD peptides and cargoes (Wadia, Stan & Dowdy, 2004; Futaki, 2005; Kaplan, Wadia & Dowdy, 2005; Wadia & Dowdy, 2005; Chang et al., 2007).

To sum it all, the phenomenon of NPT mediated by AID peptides is common in animal and plant cells. However, no study reveals that there are other species possessing this NPT phenomenon except within animals and plants. Screening different species in order to determine NPT among organisms is mandatory because some researchers have proposed that the AID peptide entry mechanism was by lipid raft-dependent macropinocytosis (Nakase et al., 2004). The ingestion route or intracellular transport through the plasma membrane can be a point of classification in taxonomy that is usually applied to evolutionary relationships. Based on this study, we deduce a new perspective on evolution.

## Materials and Methods

### Plasmid Constructions, Protein Expression and Peptide Synthesis

pR9, pQE8-GFP, pRFP and pR9-GFP plasmids were described previously (Elowitz et al., 1999; Chang et al., 2005a, 2007; Wang et al., 2006; Hou et al., 2007). These plasmids contain the six-histidine residue (6His)-tagged nona-arginine (R9), GFP, RFP and R9-GFP, respectively, and are under the control of the T7 promoter. R9 peptide, GFP, RFP as well as R9-GFP fusion protein were overexpressed and purified from *Escherichia coli* as described previously (Chang et al., 2005a; Chang, Hsu & Lee, 2005b; Chang & Lee, 2005). Synthetic nona-arginine (SR9) peptide reaching >95% purity was described previously (Wang et al., 2006; Chang et al., 2007).

### Cell Culture

#### *Cyanobacteria*

Both *Synechocystis* sp. PCC 6803 (ATCC, Manassas, VA; 27184) and *Synechococcus elongatus* PCC 7942 (ATCC, 33912) strains of cyanobacteria (blue-green algae) were kindly provided by Dr. Yuh-Jang Shieh (Weng & Shieh, 2004). They were grown in BG-11 medium (17.65 mM $NaNO_3$, 0.23 mM $K_2HPO_4$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 0.24 mM $CaCl_2 \cdot 2H_2O$, 0.02 mM trisodium citrate, 0.02 mM $FeCl_3 \cdot 6H_2O$, 0.003 mM EDTA-$Na_2Mg$ salt, 0.19 mM $Na_2CO_3$ and 0.1% trace metal mix A5) with mild shaking at 50 rpm and regular illumination at 28°C (Stork et al., 2005).

#### *Bacteria*

Both *E. coli* DH5α (Invitrogen, Carlsbad, CA) and *Arthrobacter ilicis* D-50 (Chou & Huang, 2005) strains were cultured in 3 ml of Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 170 mM NaCl [pH 7.5]) at 37°C without any antibiotics and maintained at optical density at 600 nm ($OD_{600}$) was 0.5 as previously described (Chang et al., 2005b).

#### *Archaebacteria*

*Thermus aquaticus* (ATCC, 25105) was precultured with #461 broth (50% double-strength Castenholz salts and 0.1% tryptone and yeast extract (TYE) [pH 7.6]; ATCC) in tubes at 70°C. The incubated tubes were within a closed jar with a moistened paper, which was added to maintain humidity. Once growth was obtained in broth, 200 µl of cells were transferred into a new flask with fresh #461 broth and culturing continued.

#### *Green algae*

The method of the chlorophyte (*Chlorella vulgaris* Beij., kindly provided by Dr. Jiunn-Tzong Wu) culture was the same as that of the cyanobacteria culture. Chlorophytes were isolated from Lake Li-yu in Taiwan (Wu et al., 2006).

#### *Fungi*

A little piece of *Antrodia cinnamomea* (ATCC, 200183) was cut from its carpophores and put on the MEA (2% maltose extract, 2% glucose, 0.1% peptone, 2% agar) plates aerobically as previously described (Lin, Wu & Chou, 2006). This tissue was incubated at 28°C hermetically.

*Yeasts*

Inoculated Y187 yeast (*Saccharomyces cerevisiae*) cells were cultured in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C overnight (Clontech, Mountain View, CA). This culture was transferred into a flask containing fresh YPD medium and incubated at 30°C with shaking at 200 rpm until $OD_{600}$ reached 0.5. Then, cells were ready to be utilized in experiments.

NPT

NPT was performed as described previously (Wang et al., 2006; Chang et al., 2007; Liu et al., 2007). In brief, cells of *E. coli* DH5α, cyanobacteria or archaea were treated with either GFP or RFP at a final concentration of 800 nM after medium was aspirated as a control. Green algae were prepared at a different final concentration because of the coordination of experimental groups. *Antrodia cinnamomea* was treated with 4 μM of GFP or 3 μM of RFP; 5 μM of GFP was applied in yeast cells. All of them were treated for 20 min at room temperature unless otherwise specified. To test NPT, fluorescent proteins were premixed with AID peptides including R9 and SR9 at different molecular ratios and then transferred into different kinds of cells. After 20 min, these mixtures were removed, and cells were washed with double-deionized water for twice in order to remove any free peptides and proteins which might interfere with experiments. All energy-dependent molecular movement in the cell membrane was essentially arrested in live cells at 4°C (Vives, Brodin & Lebleu, 1997). Various endocytic inhibitors—e.g., 2 mM of *N*-ethylmaleimide (NEM), which was used to inhibit the caveolae pathway; 2 μM of valinomycin and nigericin, which were ionophores used to neutralize endosomal pH; and 10 mM of sodium azide, which was used to induce ATP depletion—as well as macropinocytic inhibitors—e.g., 100 μM of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), which was known to block the formation of macropinosomes, and 5 μM of cytochalasin D (CytD), which was used to disrupt actin filaments—were described previously (Chang et al., 2005a, 2007). ß-Galactosidase (ß-Gal) analysis in bacteria was described previously (Chang et al., 2007).

Flow Cytometry

Flow-cytometric analysis was conducted using the Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA) with FL1 filters (excitation 488 nm, emission 525 nm) for GFP detection. Samples were counted and analyzed with CXP software as described previously (Wang et al., 2006).

Fluorescent and Confocal Microscopy

Images were observed under an Eclipse E600 fluorescent microscope (Nikon, Melville, NY) and recorded by a Penguin 150CL cooled charge-coupled device camera (Pixera, Los Gatos, CA). Green and red fluorescent images were observed using the TCS SL confocal microscope system (Leica, Wetzlar, Germany), and relative intensities of fluorescent images were quantified by UN-SCAN-IT software (Silk Scientific, Orem, UT) as described previously (Chang et al., 2005a; Wang et al., 2006).

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. Means and SDs were calculated for each sample assayed at least in triplicate. The levels of statistical significance were set at $P < 0.05$ (*) or 0.01 (**).

## Results

Protein Transduction in Cyanobacteria

Based on sequencing studies of the ribosomal RNA genes, all living organisms could be divided into three phylogenetic groupings: Bacteria, Archaea and Eukarya (Woese & Fox, 1977). All prokaryotes with peptidoglycan in the plasma membrane belonged to the domain Bacteria, including *E. coli* and cyanobacteria (Fig. 1). In contrast, prokaryotes without peptidoglycan belonged to the second domain, Archaea. The remaining organisms were classified in the third domain, Eukarya. Here, we chose cyanobacteria and *E. coli* from Bacteria, thermophiles from Archaea and green algae, fungi and yeast from Eukarya to study whether NPT might be conducted in these species.

Before NPT analysis, some pitfalls needed to be avoided. First, we tested whether the cyanobacterial cells (blue-green algae) could emit any fluorescence by themselves when stimulated by different excited light. Both *Synechocystis* sp. PCC 6803 (Fig. 2a–d, i, j) and *Synechococcus elongatus* PCC 7942 strains of cyanobacteria (Fig. 2e–h, k, l) emitted strong red fluorescence when stimulated by blue (Fig. 2b, f) or green (Fig. 2c, g) light using a confocal microscope at a magnification of ×1,000. In contrast, dead cyanobacteria would not emit any signal after stimulation by green light (Fig. 2d, h). These results indicated that cyanobacteria possessed red autofluorescence and RFP could not serve as a trace marker in protein transduction studies.

Second, macromolecules, such as GFP, could be fed into both PCC 6803 and 7942 cells in the control group treated

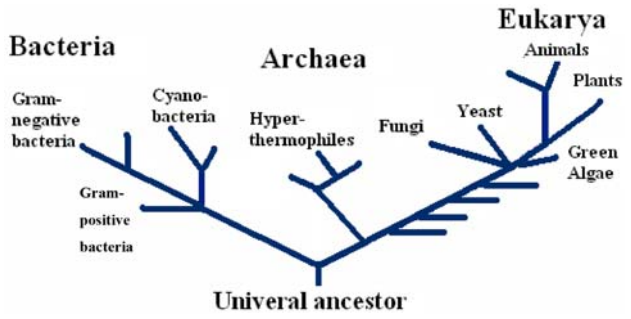


**Fig. 1** The three-domain system. All living beings are divided into three groups: Bacteria, Archaea and Eukarya (Woese & Fox, 1977). Cyanobacteria and *E. coli* from Bacteria, thermophiles from Archaea and green algae, fungi and yeast from Eukarya were chosen in the present study

with GFP (Fig. 2i, k) as well as in the experimental group treated with R9-plus-GFP mixture (Fig. 2j, l). These results confirmed that uptake of proteins by prokaryotic cyanobacteria involves classical endocytosis (Kroth, 2002). Accordingly, we chose some physical and chemical endocytosis inhibitors, including low temperature, NEM, valinomycin, nigericin and sodium azide (Chang et al., 2005a, 2007) to treat cyanobacterial cells (Fig. 3a, g). Our results revealed that all endocytic inhibitors decreased the admissibility of GFP in both PCC 6803 (Fig. 3b–f) and 7942 (Fig. 3h–l) cells, and less green fluorescence was detected by a confocal microscope. Furthermore, cells were still alive and emitted red fluorescence continually after the treatment of these inhibitors (data not shown). Relative intensities of fluorescence were quantified by the UN-SCAN-IT software (Fig. 3m). Among endocytic inhibitors, NEM showed the best efficiency of endocytic inhibition in both strains of cyanobacteria.

In order to find out the best timing that was both effective for endocytic inhibition and harmless to survival in cells, we devised different time courses of endocytic inhibitor treatment. NEM was chosen for this experiment since it was the most effective inhibitor for the endocytic uptake of GFP by both cyanobacteria strains (Fig. 3m). Transparently, both PCC 6803 (Fig. 4a–f) and 7942 (Fig. 4g–l) cells remained alive in different time courses of NEM treatment (Fig. 4m). One-minute treatment of NEM was shown to be harmless and sufficient to inhibit the endocytic pathway in both strains (Fig. 4m).

Both PCC 6803 and 7942 cells were treated with either GFP only or R9-plus-GFP mixture after NEM treatment for 1 min. No or little green fluorescence was detected in the two strains of cells treated with GFP alone (Fig. 5a, c). This indicated that GFP did not pass through the cell membrane and that cells were not injured (data not shown for red fluorescence). Contrarily, cells treated with R9-plus-GFP mixture exhibited remarkable green fluorescence (Fig. 5b, d), suggesting that NPT indeed occurred in cyanobacteria.

To further investigate whether AID facilitated protein delivery into cells by macropinocytosis in cyanobacteria, we chose some macropinocytic inhibitors, including EIPA

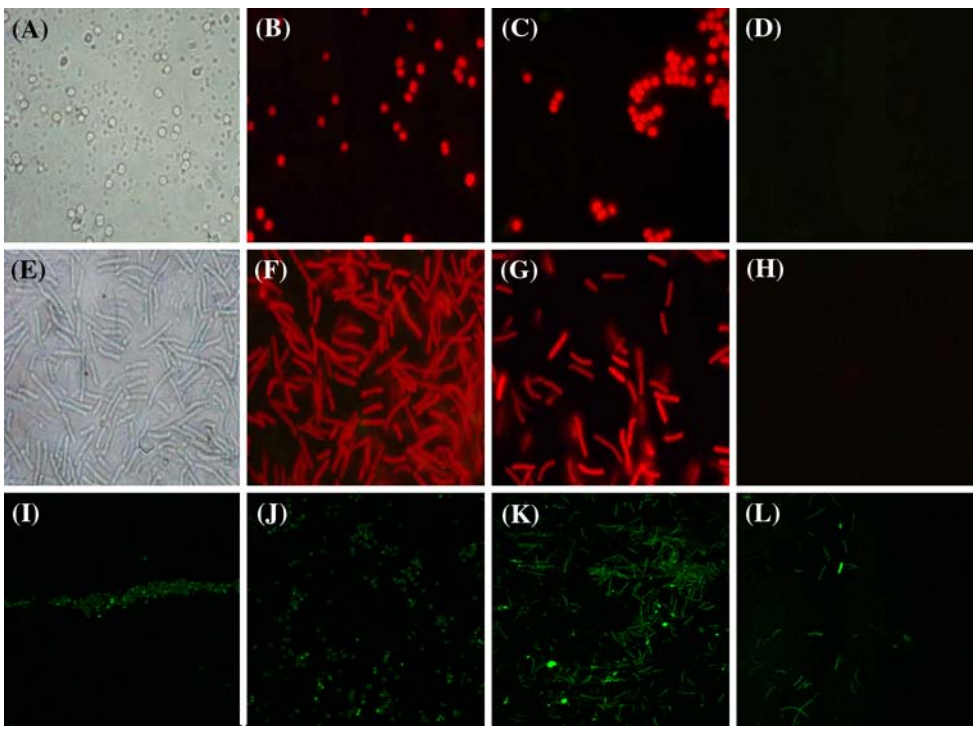


**Fig. 2** Autofluorescence and endocytosis of cyanobacteria. The PCC 6803 strain of cyanobacteria was observed in visible light (**a**) under an Eclipse E600 fluorescent microscope and in blue (**b**) or green (**c**) light by the TCS SL confocal microscope system. This strain was sterilized with 70% alcohol and observed in green light (**d**). The PCC 7942 strain of cyanobacteria was observed in visible (**e**) and in blue (**f**) or green (**g**) light under a confocal microscope at a magnification of $\times$1,000. Dead cells of this strain were observed in green light (**h**). PCC 6803 was treated with either 1.8 μg of GFP (**i**) or R9-plus-GFP mixture (**j**) at the molecular ratio of 4.7:1 (3.7 μg of R9 and 1.8 μg of GFP), while PCC 7942 was treated with either GFP (**k**) or R9-plus-GFP (**l**)

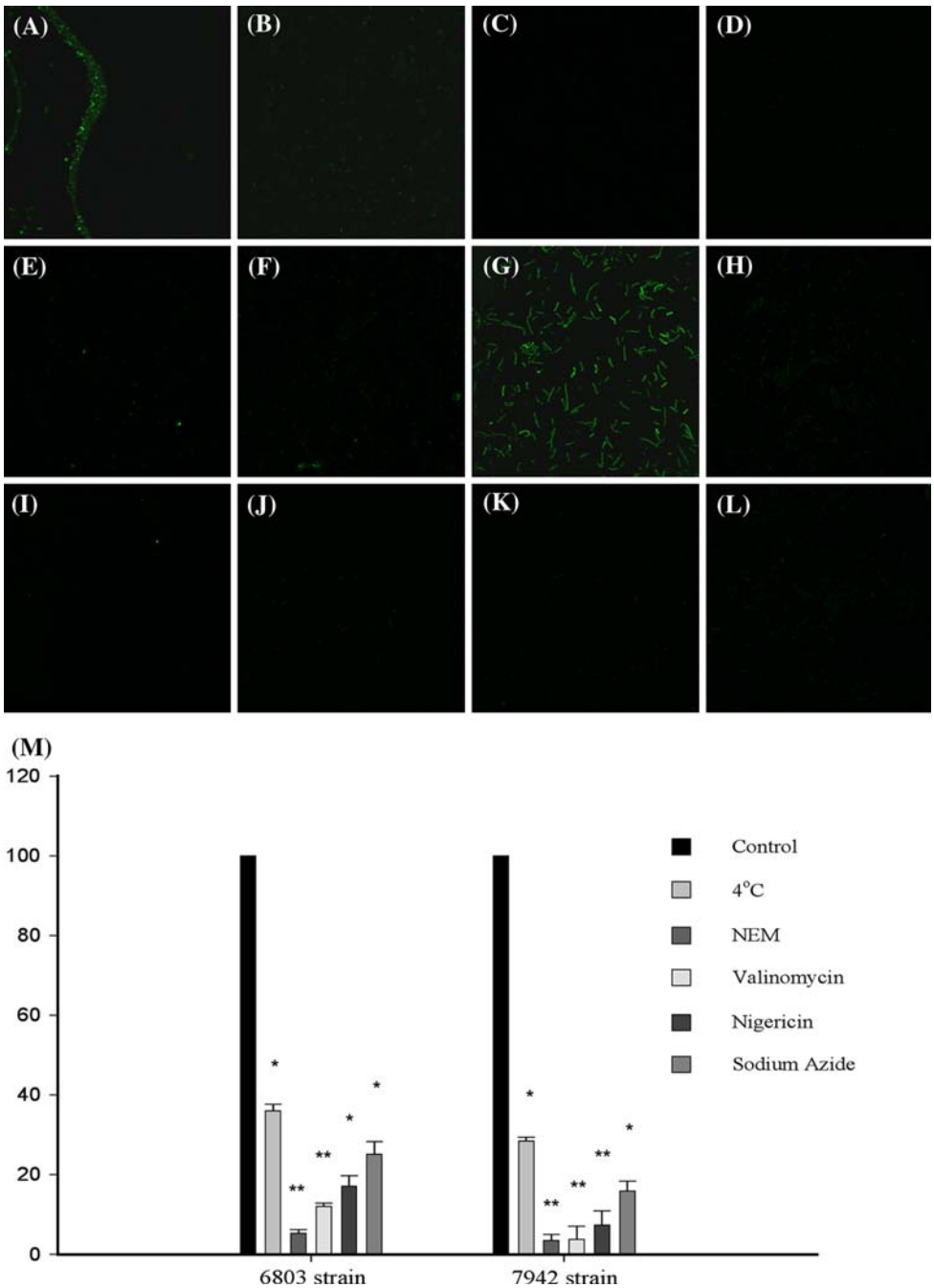


**Fig. 3** Effect of endocytic inhibitors in cyanobacteria. The PCC 6803 strain of cyanobacteria was treated with 1.8 μg of GFP only at room temperature (**a**) as a control, at 4°C (**b**) or at room temperature in the presence of 2 mM of NEM (**c**), 2 μM of valinomycin (**d**), 2 μM of nigericin (**e**) or 10 mM of sodium azide (**f**). The PCC 7942 strain of cyanobacteria was treated with GFP only at room temperature (**g**) as a control, at 4°C (**h**) or at room temperature in the presence of 2 mM of NEM (**i**), 2 μM of valinomycin (**j**), 2 μM of nigericin (**k**) or 10 mM of sodium azide (**l**). Results were observed at a magnification of ×1,000 using a confocal microscope. Relative endocytic efficiencies were quantified and compared in the absence or presence of different inhibitors (**m**). Significant differences ($*P < 0.05$ and $**P < 0.01$) between control and experimental groups are marked

and CytD. Although cells were pretreated with NEM, which could stop endocytosis, R9 peptide still could bind to GFP and pass through the cells (Fig. 6a, d). Nevertheless, NPT mediated by R9 peptide decreased in the presence of macropinocytic inhibitors (Fig. 6b, c, e, f). NPT in PCC 6803 cells was most constrained by CytD (Fig. 6g). However, NPT was highly inhibited by EIPA in PCC 7942 cells (Fig. 6g). These results indicate not only that NPT exists in the domain Bacteria but also that the mechanism of NPT should involve macropinocytosis.

## Protein Transduction in Bacteria

Fluorescent proteins including RFP and GFP were mixed with R9 peptide first and then transferred by NPT into gram-negative DH5α bacterial cells for 20 min. After removal of the original medium, *E. coli* cells were washed with double-deionized water. Several bacteria were dropped on slides and observed using a confocal microscope at a magnification of ×1,000. No signal could be detected in bacteria treated with RFP only when green light was utilized to stimulate cells (Fig. 7a). However, bacteria treated

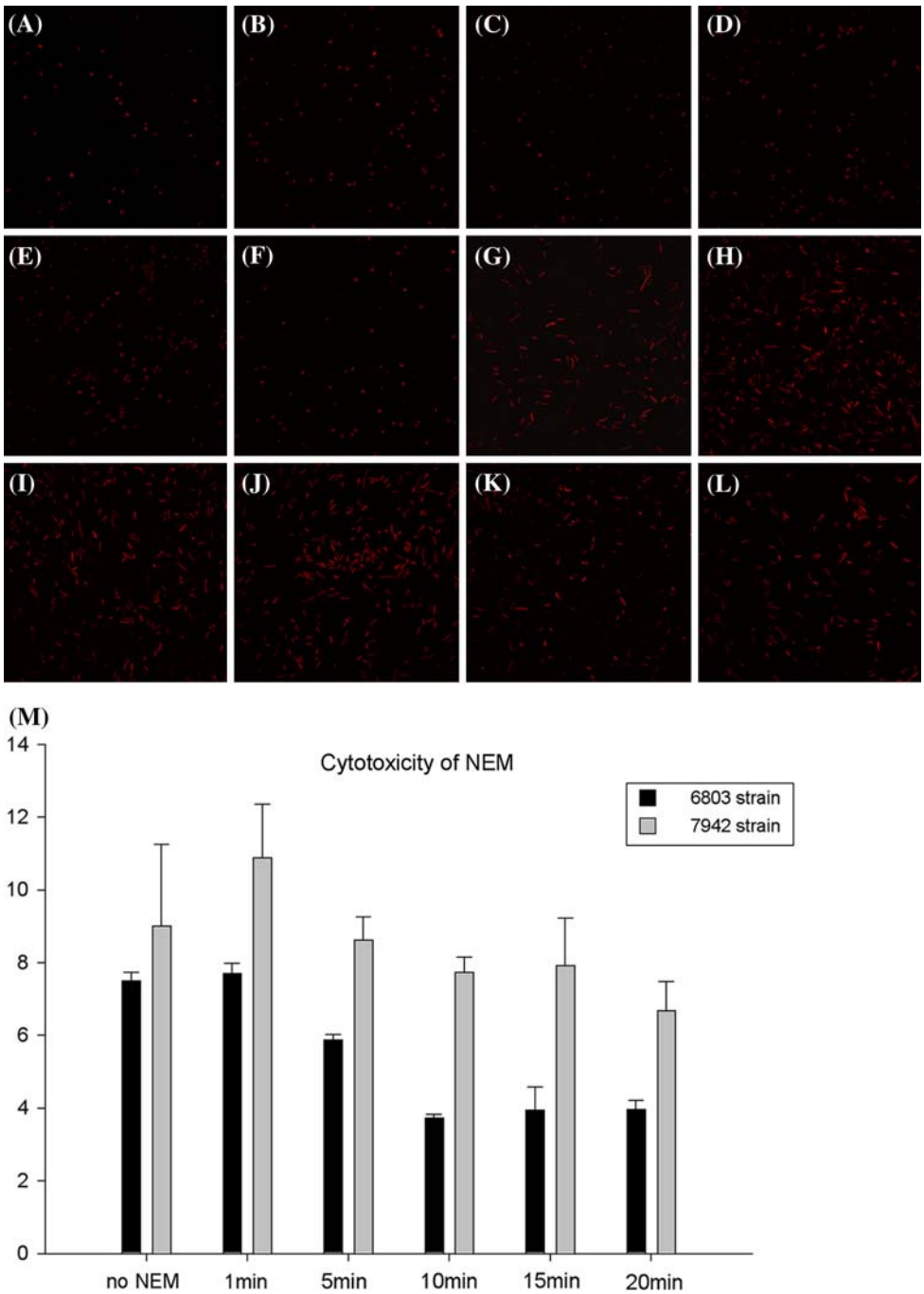


**Fig. 4** Cytotoxicity of NEM in cyanobacteria. Both PCC 6803 (**a**–**f**) and 7942 (**g**–**l**) strains of cyanobacteria were treated without or with 2 mM of NEM for different time courses and observed in green light at a magnification of ×1,000 using a confocal microscope. The time courses were set without NEM (**a**, **g**) or with NEM at 1 (**b**, **h**), 5 (**c**, **i**), 10 (**d**, **j**), 15 (**e**, **k**) and 20 (**f**, **l**) min. Relative fluorescence intensities at different time courses were analyzed (**m**)

with R9-plus-RFP mixture displayed red fluorescence (Fig. 7b). Under the same conditions as described above, bacteria treated with R9-plus-GFP mixture exhibited green fluorescence, while cells treated with GFP did not show any signal in controls (data not shown). To demonstrate that R9 peptide can carry any kind of protein into bacteria, fluorescent proteins were replaced by $\beta$-Gal enzyme and $\beta$-Gal activity was assayed. The same result was obtained as described above (data not shown). To sum it all, these results suggested that R9 peptide was capable of carrying other cargo proteins through the cell membrane and NPT might exist in gram-negative bacteria.

On the other hand, gram-positive *Arthrobacter ilicis* D-50 bacteria were bacillary types and colorless observed in bright field using a confocal microscope at a magnification of ×1,000 (Fig. 7c). Besides, there was no emission of fluorescence from cells when they were excited by green light using a confocal microscope (Fig. 7d). Cells

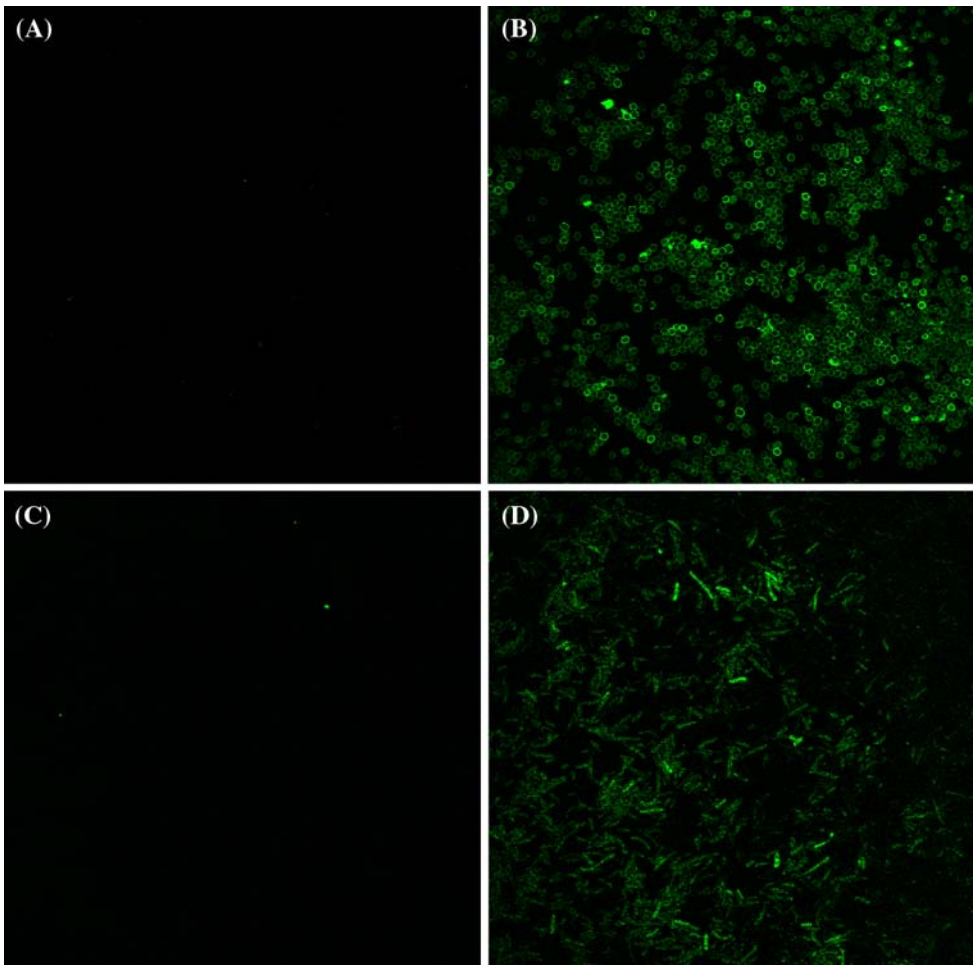


**Fig. 5** NPT in cyanobacteria in the presence of NEM. The PCC 6803 strain of cyanobacteria was treated with either 1.8 μg of GFP alone (**a**) or R9-plus-GFP mixture (**b**) at the molecular ratio of 4.7:1 in the presence of 2 mM of NEM for 1 min in order to restrain the endocytic pathway. The PCC 7942 strain of cyanobacteria was treated with either GFP alone (**c**) or R9-plus-GFP mixture (**d**) in the presence of NEM. Results were observed at a magnification of ×1,000 using a confocal microscope

would not show any fluorescence when treated with GFP (Fig. 7e). In contrast, bacteria treated with SR9-plus-GFP mixture displayed green fluorescence (Fig. 7f). Although the properties and structures of gram-positive bacteria are quite different from those of gram-negative bacteria, there was proof that NPT also existed in *A. ilicis* D-50 bacteria.

## Protein Transduction in Archaebacteria

According to the three-domain system, archaebacteria belong to the domain Archaea, which are much closer to the domain Eukarya than to the domain Bacteria (Woese & Fox, 1977). We examined whether protein transduction would appear in archaebacteria. We chose *Thermus aquaticus*, one species classified in thermophiles of archaea (Fig. 8a). Archaebacteria do not emit any fluorescence when excited under either a fluorescent or a confocal microscope (Fig. 8b). No or little signal was detected in cells treated with RFP (Fig. 8c). However, archaebacteria treated with R9-plus-RFP displayed significant red fluorescence (Fig. 8d). These results demonstrated that NPT also occurred in archaebacteria.

## Protein Transduction in Green Algae

Chlorophytes (green algae) belong to the domain Eukarya and are thought to be the ancestors of plants growing on the land (Woese & Fox, 1977). Green algae (Fig. 9a) possessed red autofluorescence when stimulated by green light (Fig. 9b) but not by blue light (Fig. 9c), using a confocal microscope at a magnification of ×1,000. However, dead green algae (Fig. 9d) could not emit enough signal when stimulated by either green (Fig. 9e) or blue (Fig. 9f) light. Therefore, RFP could not serve as a trace marker in protein transduction studies.

In the study of NPT, we found with surprise that no signal was detected in green algae treated with GFP alone (Fig. 9g), R9-plus-GFP (Fig. 9h) or SR9-plus-GFP (Fig. 9i). In contrast, algae treated with covalent R9-GFP fusion protein showed green fluorescence around cells in

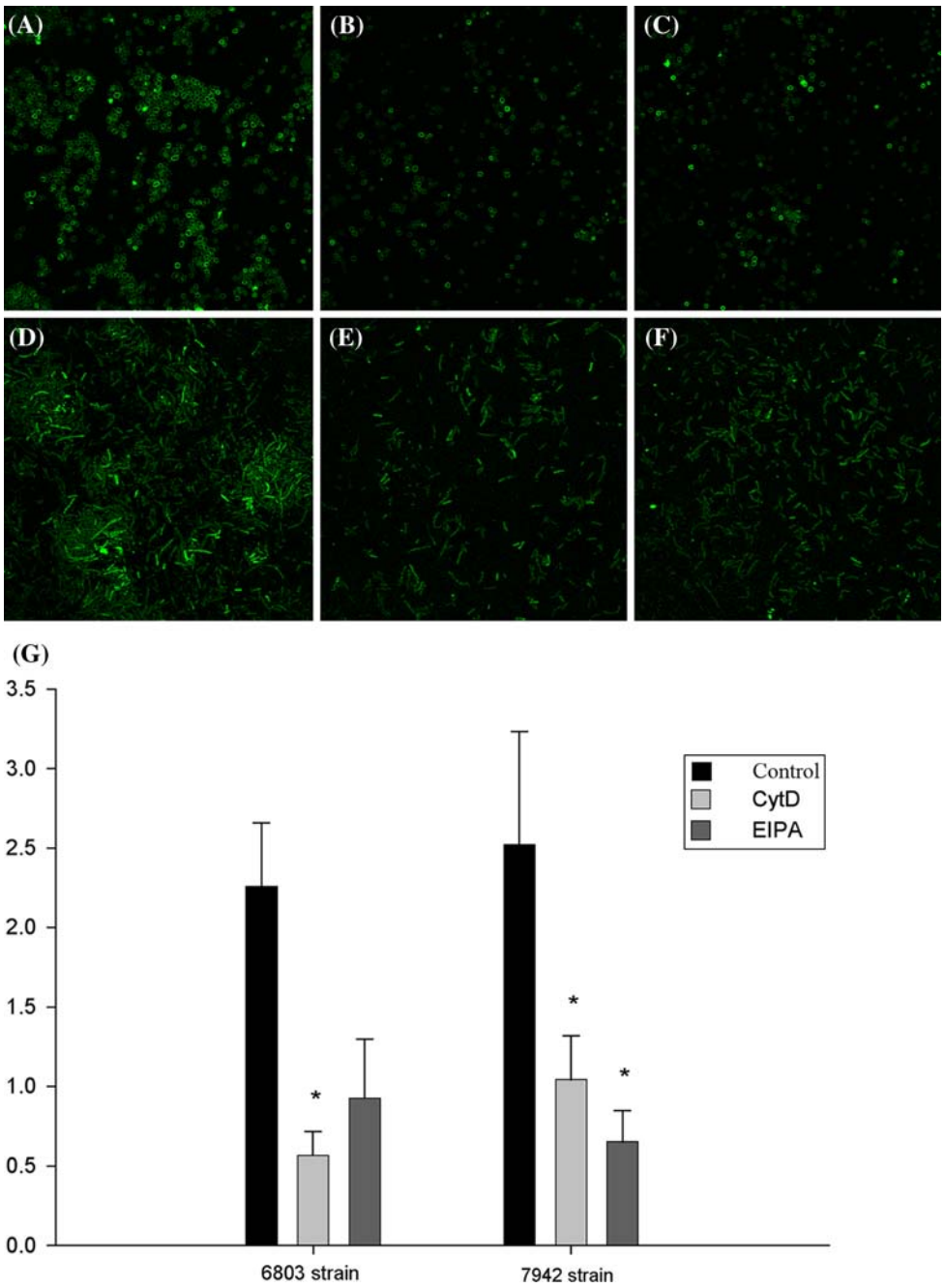


**Fig. 6** Effect of macropinocytic inhibitors on NPT in cyanobacteria in the presence of NEM. Both PCC 6803 (**a**–**c**) and 7942 (**d**–**f**) strains of cyanobacteria were treated with R9-plus-GFP mixture at the molecular ratio of 4.7:1 (1.87 μg of R9 and 897 ng of GFP) in the presence of 2 mM of NEM for 1 min (**a**, **d**) and additionally in the presence of 100 μM of EIPA (**b**, **e**) or 5 μM of CytD (**c**, **f**). Results were observed at a magnification of ×1,000 under a confocal microscope. Relative fluorescence intensities in the absence or presence of different macropinocytic inhibitors were analyzed (**g**). Significant differences ($*P < 0.05$) between control and experimental groups are marked

horseshoe shapes (data not shown). These results indicated that NPT did not exist in green algae.

## Protein Transduction in Fungi

*Antrodia cinnamomea* is a medicinal fungus (Fig. 10a) that grows naturally inside the *Cinnamonum kanehirae* trunk, a native tree species of Taiwan (Lin et al., 2006). Fungi do not emit any fluorescence when excited by green (Fig. 10b) or blue (Fig. 10c) light using a confocal microscope. No signal was observed in fungi treated with GFP alone (Fig. 10d). Fungi treated with R9-plus-GFP mixture did not show green fluorescence either (Fig. 10e), although a false spot appeared. These results indicated that NPT might not happen in fungi.

## Protein Transduction in Yeasts

Y187 yeast cells (*Saccharomyces cerevisiae*) were cultured in YPD medium at 30°C and observed in visible (Fig. 11a, c, e, g) or blue (Fig. 11b, d, f, h) light using a confocal microscope. No signal was detected in untreated yeast

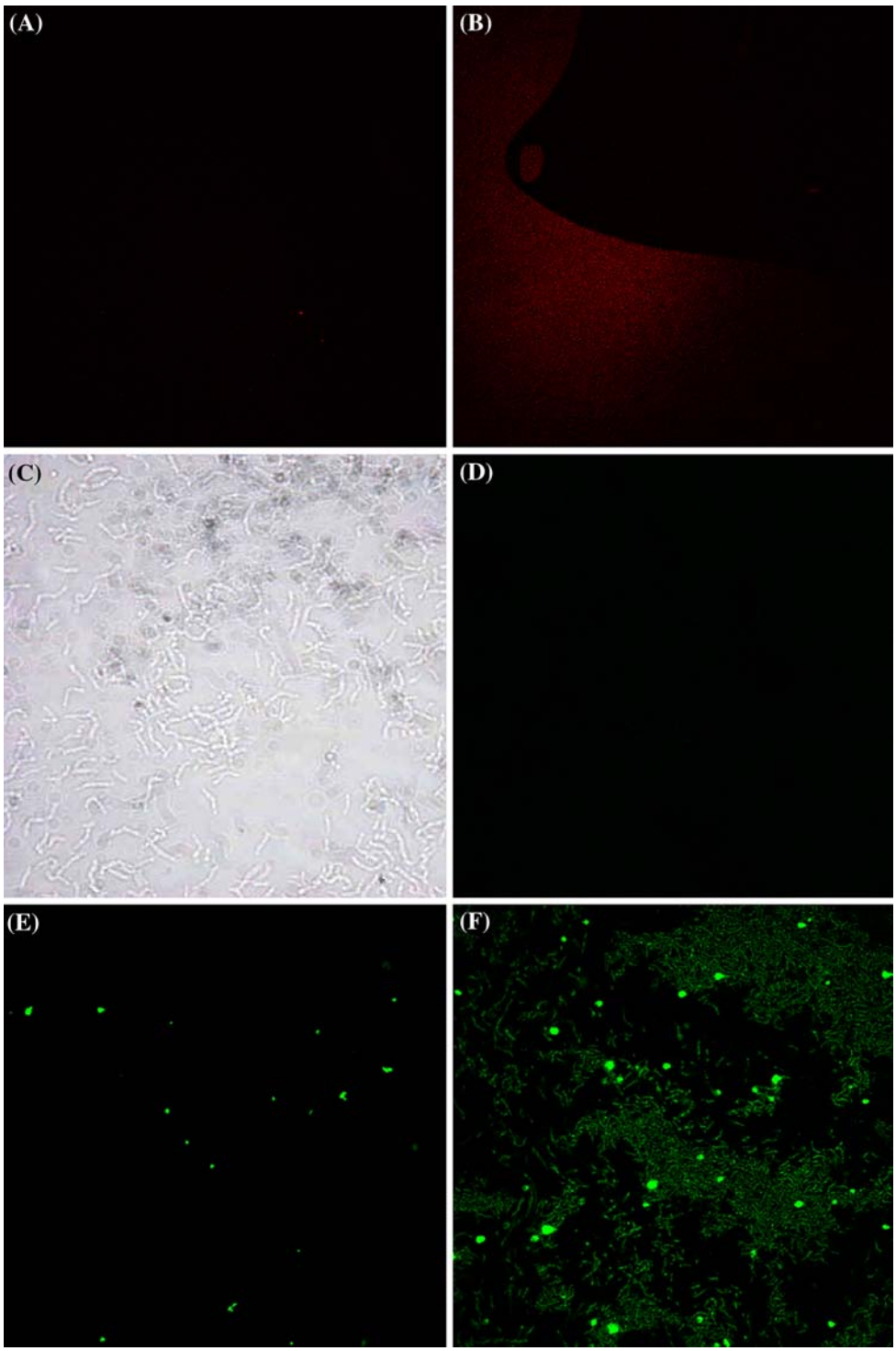


**Fig. 7** NPT in bacteria. Gram-negative DH5α bacteria were treated with either 1.2 μg of RFP only (**a**) or R9-plus-RFP mixture (**b**) at the molecular ratio of 4.5:1 (2.4 μg of R9 and 1.2 μg of RFP) for 20 min. Results were observed at a magnification of ×1,000 using a confocal microscope. Gram-positive D-50 bacteria were observed in visible (**c**) or green (**d**) light under a microscope. D-50 bacteria treated with 3.6 $\mu$M of GFP only (**e**) or SR9-plus-GFP mixture (**f**) at the molecular ratio of 3:1 (10.8 $\mu$M of SR9 and 3.6 $\mu$M of GFP) were observed at a magnification of ×1,000 under a confocal microscope

(Fig. 11b) or yeast treated with GFP alone (Fig. 11d). However, yeast treated with either R9-plus-GFP (Fig. 11f) or SR9-plus-GFP (Fig. 11h) displayed green fluorescence. These results suggested that NPT occurred in yeast.

To compare efficiency between covalent transduction and NPT, GFP internalization was analyzed in yeast by flow cytometry. No or little green fluorescence was detected in Y187 (Fig. 12a) and cells treated with GFP alone (Fig. 12b). In contrast, yeast treated with either R9-plus-GFP (Fig. 12c) or SR9-plus-GFP (Fig. 12d) exhibited green fluorescence to certain degrees in an arbitrarily selected fluorescence gate region (FL1 > 1). These fluorescence-positive yeast accounted for 8.9 ± 0.5% and 10.2 ± 1.5% of the total population for R9-plus-GFP and

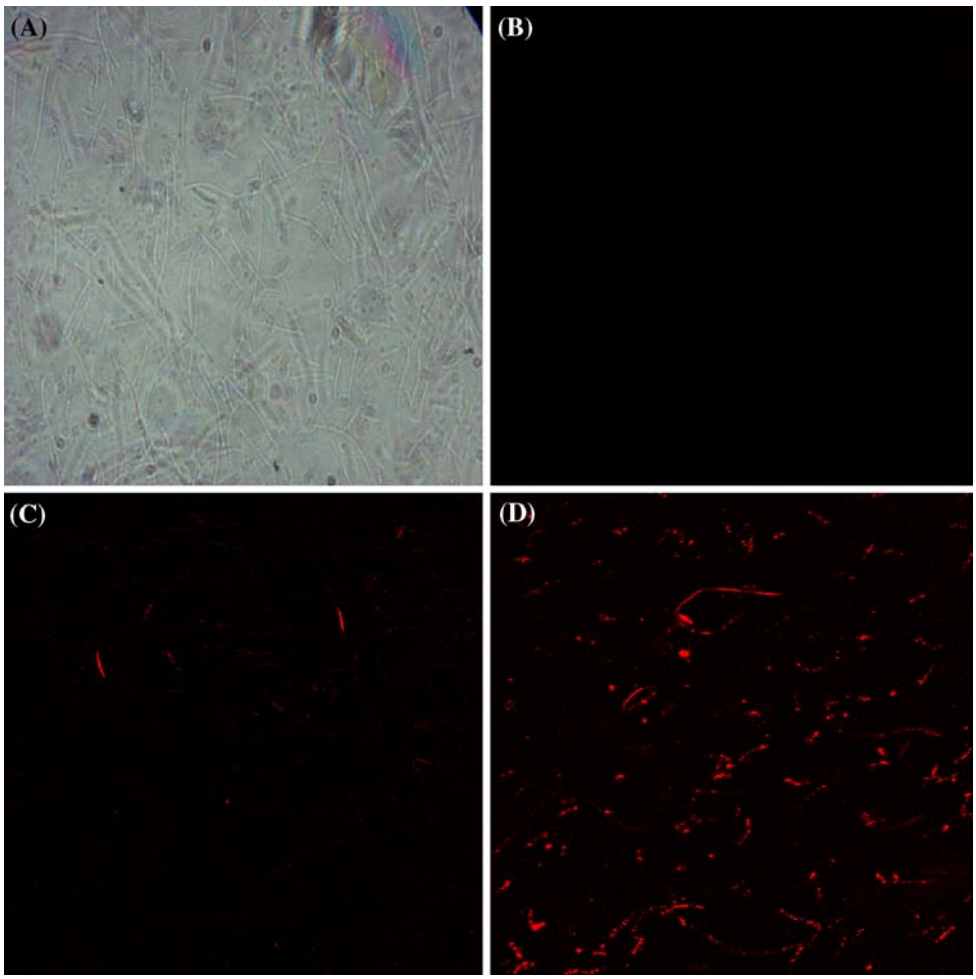


**Fig. 8** NPT in archaebacteria. Thermophiles were observed in visible (**a**) or green (**b**) light under a microscope. Archaea treated with either 1.2 μg of RFP only (**c**) or R9-plus-RFP mixture (**d**) at the molecular ratio of 4.5:1 (2.4 μg of R9 and 1.2 μg of RFP) for 20 min were observed at a magnification of ×1,000 using a confocal microscope

SR9-plus-GFP treatment, respectively (Fig. 12e). These results were consistent with those of confocal microscopy, indicating that AID peptides were effective carriers for intracellular delivery of protein in yeast.

## Discussion

We demonstrated that NPT occurs in cyanobacteria and bacteria from Bacteria, thermophiles from Archaea and animals, plants as well as unicellular yeast from Eukarya according to the three-domain system (Woese & Fox, 1977; Woese, Kandler & Wheelis, 1990). In contrast, NPT could not occur in chlorophytes (green algae) and multicellular fungi from Eukarya (Table 1). Chlorophytes belong to the domain Eukarya and are thought to be the ancestors of plants growing on the land (Woese & Fox, 1977). It is evolutionarily significant for protein transduction because green algae were the transitionary type from the cyanobacteria to the higher-level plants. Based on results of NPT, it should be an interesting clue when examining the phylogeny of chlorophytes and multicellular fungi. Previous studies proposed that chlorophytes and fungi could be grouped as a new classification of Chlorophyta (Zoochlorellae), which might derive from the phylogenetic branch point before the formation of Bacteria, Archaea, and Eukarya (Romeike et al., 2002; Lewis & Muller-Parker, 2004).

The mechanism of protein transduction across membrane barriers by AID peptides has been a topic of controversy thus far. Recent reports have demonstrated that protein transduction involves macropinocytosis, a lipid raft–dependent, receptor-, caveolae- or clathrin-independent type of endocytosis (Nakase et al., 2004; Wadia et al., 2004; Futaki, 2005; Kaplan et al., 2005; Wadia & Dowdy, 2005; Chang et al., 2007). Our results obtained from the application of classical endocytic modulators, including NEM, valinomycin, nigericin and sodium azide, are in agreement with previous studies (Nakase et al., 2004, Kaplan et al., 2005), indicating NPT does not involve classical endocytosis in cyanobacteria (Fig. 3). NPT was unaffected by treatment at 4°C, suggesting that protein entry was in a classical energy-independent pathway in cyanobacteria. Moreover, using the macropinocytosis

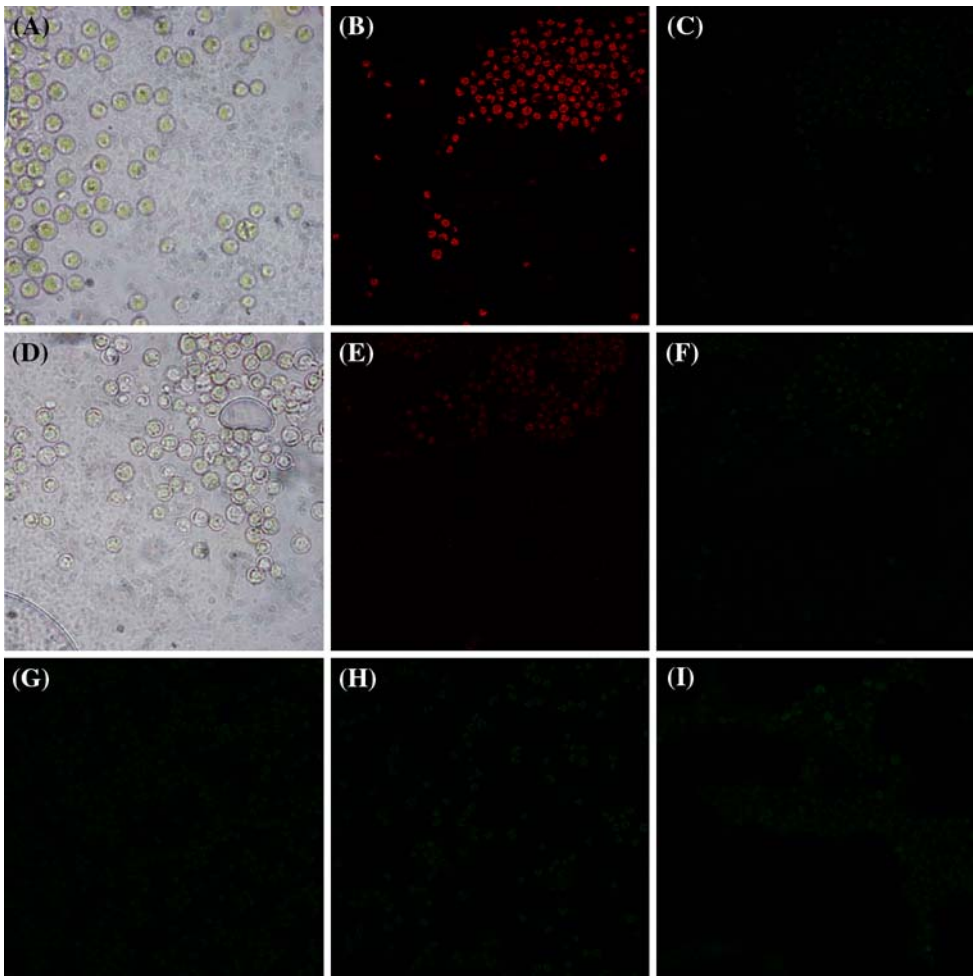


**Fig. 9** NPT in green algae. Chlorophytes were observed in visible (**a**), green (**b**) or blue (**c**) light under a microscope. Cell were sterilized with 70% alcohol and observed in visible (**d**), green (**e**) or blue (**f**) light. Chlorophytes treated with 4 μg of GFP (**g**), R9-plus-GFP mixture (**h**) at a molecular ratio of 10:1 or SR9-plus-GFP mixture (**i**) at a molecular ratio of 3:1 were observed at a magnification of ×1,000 using a confocal microscope

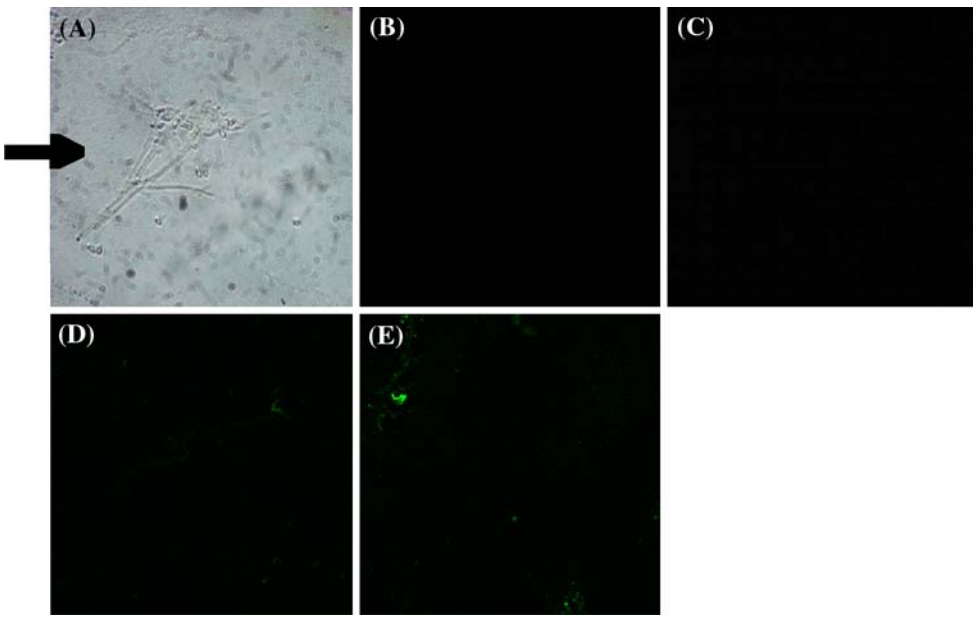


**Fig. 10** NPT in fungi. Fungi were observed in visible (**a**), green (**b**) or blue (**c**) light under a microscope. Cells treated with either 1.4 μg of GFP (**d**) or R9-plus-GFP mixture (**e**) at a molecular ratio of 4.15:1 (2.8 μg of R9 and 1.4 μg of GFP) were observed at a magnification of ×400 under a confocal microscope. A false positive appeared in the left area (**e**), indicated by an *arrow* in **a**

inhibitor EIPA and the F-actin polymerization inhibitor CytD, we found NPT was significantly reduced (Fig. 6). This demonstrates that the mechanism of AID-mediated NPT may involve macropinocytosis and actin reorganization in cyanobacteria. Taken together, these data are in agreement with our previous studies (Wang et al., 2006;

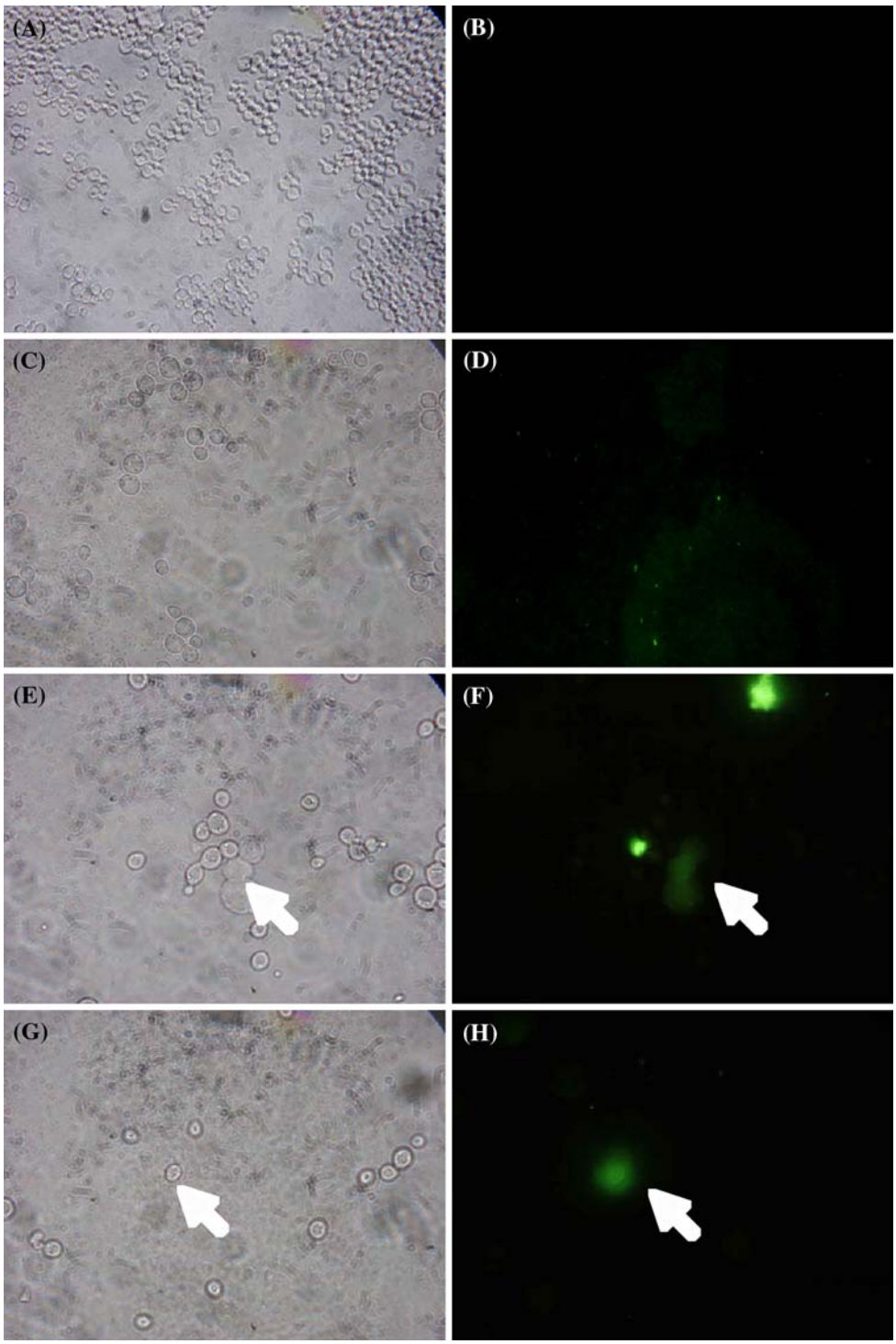


**Fig. 11** NPT in yeast. Yeast cells were observed in visible (**a**, **c**, **e**, **g**) or blue (**b**, **d**, **f**, **h**) light using a confocal microscope. Images (**a**, **b**) are shown at a magnification of ×400. Cells treated with 14.3 μg of GFP (**c**, **d**), R9-plus-GFP mixture (**e**, **f**) at a molecular ratio of 4.15:1 (28.6 μg of R9 and 14.3 μg of GFP) or SR9-plus-GFP mixture (**g**, **h**) at a molecular ratio of 3:1 (2 μg of SR9 and 14.3 μg of GFP) were observed at a magnification of ×1,000 under a confocal microscope. *Arrows* indicate corresponding positions among positive cells (**e–h**), while two false spots appeared (**f**)

Chang et al., 2007) and others using octa-arginine peptide in HeLa cells (Nakase et al., 2004).

Macropinocytosis requires actin membrane protrusions that envelope into macropinosomes (Conner & Schmid, 2003). Actin is often seen as a component in the construction of the cytoskeleton. Both green algae and fungi, which did not show the NPT phenomenon, also possess actin (Tartar et al., 2002). When macromolecules are carried into them, derived ingestion vesicles may be formed by more than two different kinds of cytoskeletons in these two species. In other words, their membrane-bound macropinosomes cannot result from actin movement only. They have to involve the participation of other cytoskeletons, such as dynamin or syndapin (Kessels & Qualmann, 2004). However, derived ingestion vesicles in other species which contained the protein transduction phenomenon,

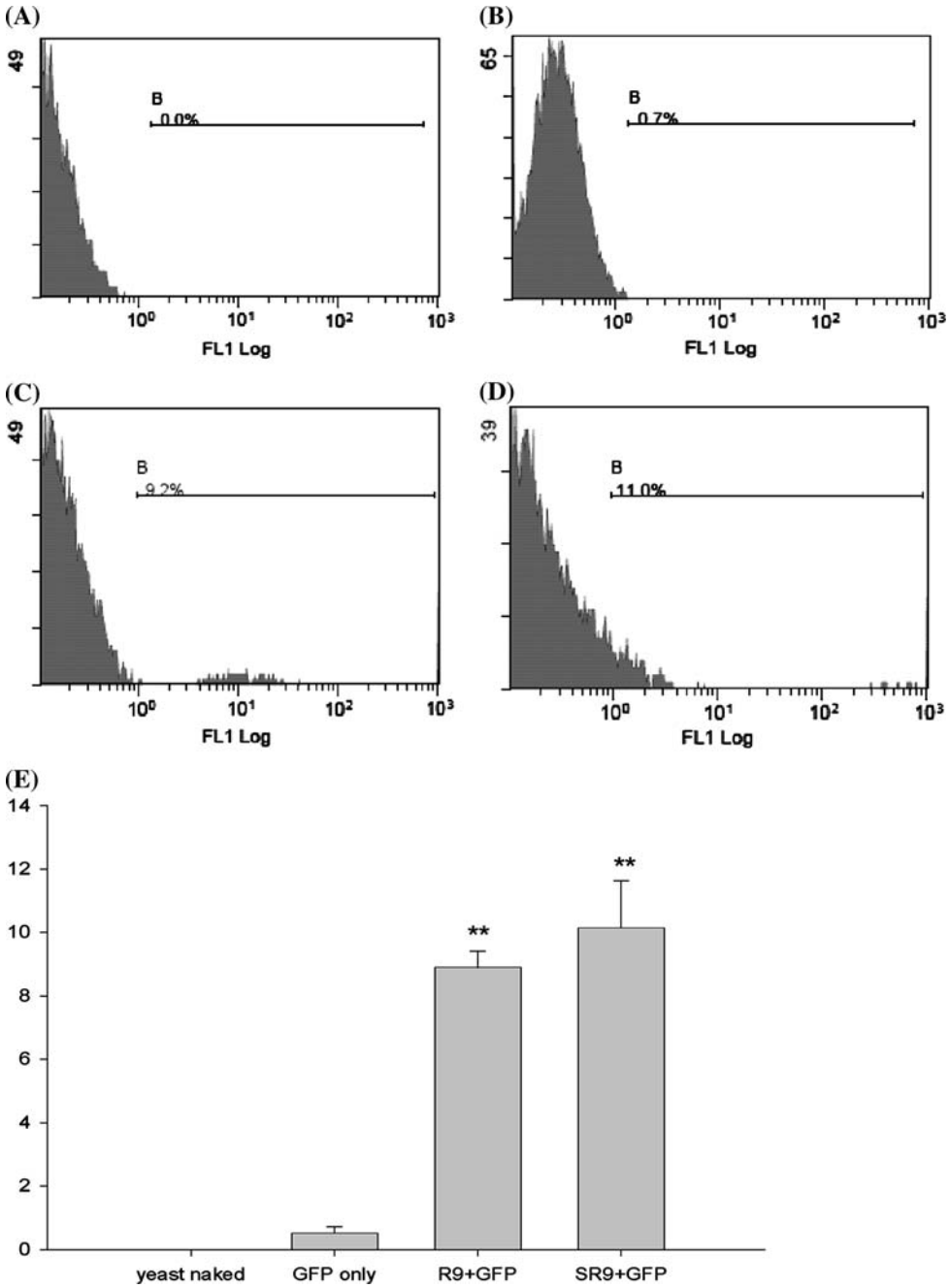


**Fig. 12** Flow-cytometric analysis of NPT in yeast. Profiles of yeast cells mock-treated as a control (**a**) or treated with 14.3 μg of GFP alone (**b**), R9-plus-GFP mixture (**c**) at a molecular ratio of 4.15:1 or SR9-plus-GFP mixture (**d**) at a molecular ratio of 3:1 are shown. Fluorescent images of yeast cells were analyzed by the Cytomics FC500 flow cytometer. Cells counts (*y* axis) at different fluorescent gate regions (*x* axis) were analyzed with CXP software. M1, mean fluorescence intensity of a population over the fluorescence gate region (FL1 > 1). (**e**) Comparative protein transduction efficiency derived from combined profiles. Data represent means ± SD of three independent experiments performed in duplicate. Significant differences (**$P < 0.01$) between control and experimental groups are marked

**Table 1** Summary of species characteristics and important findings in this study

| Species | Domain | Cell wall | Cell wall with chitin | Cell with chloroplast | Endocytosis | NPT |
|---|---|---|---|---|---|---|
| *Synechocystis* sp. PCC 6803 (cyanobacteria) | Bacteria | √ | | √ | √ | √ |
| *Synechococcus elongatus* PCC 7942 (cyanobacteria) | Bacteria | √ | | √ | √ | √ |
| *Escherichia coli* DH5α (gram-negative bacteria) | Bacteria | √ | | | | √ |
| *Arthrobacter ilicis* D-50 (gram-positive bacteria) | Bacteria | √ | | | | √ |
| Thermus aquaticus | Archaea | √ | | | | √ |
| *Chlorella vulgaris* Beij. (green algae) | Eukarya | √ | √ | √ | | |
| Antrodia cinnamomea (fungi) | Eukarya | √ | √ | | | |
| Saccharomyces cerevisiae Y187 (yeasts) | Eukarya | √ | √ | | | √ |

shown in our data with bacteria, cyanobacteria, archaea and yeasts, can employ the ambulating of actins solely. This hypothesis probably can explain the divergence we obtained in the results of six species.

The particular structure of the cell walls in different living organisms offers another disputable hypothesis for our results in protein transductions. Chitin, a crystalline polymer of *N*-acetyl-D-glucosamine, was an indispensable structural component of the cell walls in yeasts (Henar Valdivieso, Duran & Roncero, 1999), fungi and green algae (Pearlmutter & Lembi, 1978; Henar Valdivieso et al., 1999). With chitin, the density of cell walls is increased and cells are protected inside from the intrusion of other macromolecules. Without chitin, the cell wall integrity is disrupted by exposure to acetic acid (Pearlmutter & Lembi, 1978). However, it is difficult to clarify why the yeast, which also contains chitin, still allows the ingress of AID peptides. We propose two potential reasons herein. The first reason is that the presence of glycosaminoglycans (GAGs) on cell surfaces is required for protein transduction in some animal cell lines (Fawell et al., 1994; Wadia & Dowdy, 2002; Console et al., 2003). The more GAGs present on the cell surface, the more easily protein transduction happens. In addition, cell surface heparin sulfate proteoglycans can be another mediator in protein internalization. AID peptide uptake can be competitively inhibited by the degradation of heparin sulfate side chains with GAG lyases (Wadia & Dowdy, 2002). Cells rich in either heparin sulfate or GAGs possess the protein transduction phenomenon, even though yeasts are covered by positively charged chitin. Nevertheless, fungi and green algae, which have protein transduction deficiencies, may result from both effects of less heparin sulfate or GAGs and the homogeneously charged chitin repulsion.

The second reason yeasts with chitin cannot lose the function of protein transduction is that there are different chitin synthase genes involved. There are five different gene classes at least in chitin synthase, and they all differ in not only function but also catalytic properties (Henar Valdivieso et al., 1999). Class V gene in chitin synthase has been identified only in filamentous fungi, and its function is still unknown today, while others have been demonstrated in both fungi and yeasts (Henar Valdivieso et al., 1999). We hypothesize that this class V gene probably is the crucial component that causes the failure of protein internalization.

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