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Cellular uptake and subsequent intracellular trafficking of R8-liposomes introduced at low temperature

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Abstract

Intracellular trafficking is a determining factor in the transgene expression efficiency of gene vectors. In the present study, the mechanism of the cellular uptake of octaarginine (R8)-modified liposomes, when introduced at 37 °C and 4 °C, was investigated in living cells. Compared with 37 °C, the uptake of R8-liposomes was only slightly reduced at 4 °C. Dual imaging of liposomes and plasma membranes revealed that R8-liposomes were internalized by vesicular transport, and partially escaped to the cytosol at the perinuclear region at 37 °C. When introduced at 4 °C, intracellular liposomes were observed within a specific region close to the plasma membrane, and internalization of the plasma membrane was completely inhibited. Therefore, at 4 °C, R8-liposomes appear to enter cells via unique pathway, which is separate and distinct from energy-dependent vesicular transport. The subsequent nuclear delivery of encapsulated pDNA, when introduced at 4 °C, was less prominent compared with those introduced at 37 °C. Collectively, these findings demonstrate that a vesicular transport-independent pathway is responsible for the cellular uptake of liposomes. In addition, the uptake route is closely related to the subsequent nuclear delivery process; the operation of an endogenous vesicular sorting system is advantageous for the nuclear delivery of pDNA.

Keywords: Intracellular trafficking; PTD; R8; Liposome; Cellular uptake

1. Introduction

Improvement in the cellular delivery system for macromolecules is an important issue in the area of drug/gene therapy. Recent studies have revealed that protein transduction domains (PTDs) are promising devices for improving the delivery of various types of biologically active molecules [1,2] such as proteins [3–6], nucleic acids [7–10] and liposomes [11,12]. One of the most widely studied carrier peptide is derived from human immunodeficiency virus-type 1 (TAT) [13,14], which consists of 6 arginine and 2 lysine residues. Based on the high arginine content within the TAT sequence, Futaki et al. synthesized polypeptides that consist solely of arginine residue, and characterized their cellular uptake and intracellular distribution [15–17]. As a result, it was revealed that an octamer of arginine (R8) showed the most efficient internalization. In addition, studies in our laboratory showed that stearylated R8 (STR-R8) was a potent transfection device, which is capable of efficiently delivering plasmid DNA (pDNA) into the cell and even into the nucleus [10,18,19]. Moreover, concerning PTD-modified liposomes, Torchilin et al. compared the cellular uptake of certain types of liposomes, in which TAT-derived PTD was modified on the liposomes with various extents of exposure by means of polyethyleneglycol as a spacer. The findings indicated that liposomes were internalized, only when TAT was displayed on the surface of the liposome so as to readily interact with the cell membrane.

Concerning the mechanism for the uptake of PTD ser se or PTD-modified cargos, initial studies indicated that their uptake was not inhibited by incubation at low temperature, a condition in which

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all of the active cellular uptake is interrupted [11,13,15,20,21]. As a result, it was concluded that PTD-modified cargos were taken up via a vesicular transport-independent pathway (e.g., penetration).

However, a recent report indicated that fixation results in a significant overestimation of cellular uptake and reorganization of intracellular distribution [22]. Because of this, mechanism for the cellular entry has been reexamined using living cells. While current studies found that this process was energy-dependent, the mechanism of internalization of PTDs or PTD-cargos at 37 °C is, however, still controversial, presumably due to differences in the experimental conditions used. Current candidates for the uptake pathway include clathrin-mediated endocytosis [10,22–25], caveolae-mediated endocytosis [26–28] and macropinocytosis [14,17,29]. We recently demonstrated that physiochemical aspects, such as the density of the PTD was a determining factor in sorting the internalization pathway between clathrin-mediated endocytosis and macropinocytosis [30]. Furthermore, the existence of cellular uptake route at low temperature conditions is also a current topic that needs to be examined. Although some reports indicated that cellular uptake is diminished by incubation at 4 °C [10,14,22,28], R8 or heptaarginine modified with a tryptophan residue in the C-terminus (R7W) were reported to be take up by cells even at 4 °C [17,31]. Moreover, confocal images indicate that the intracellular distribution of these peptides were distinct from that taken up at 37 °C. As a result, the possibility of vesicular transport-independent internalization in the case of octaarginine or certain types of PTD peptides cannot be excluded.

The purpose of the present study was to investigate the mechanism of cellular uptake and the subsequent intracellular trafficking of R8-modified liposomes introduced at 37 °C and 4 °C in living cells. To accomplish this, we used recently developed R8-modified liposomes, which are highly associated with the plasma membrane and are efficiently taken up in an in vitro cell culture system [33]. To permit R8 to be displayed on the surface of liposome, it was stearylated, and was then anchored to lipid vesicles via its stearyl moiety. In addition, a condensed pDNA was encapsulated into the R8-liposomes so as to quantitatively evaluate subsequent nuclear delivery by means of TaqMan PCR, in an attempt to address the relationship between the intracellular fate and transgene-expression efficiency.

2. Materials and methods

2.1. General

Cholesteryl hemisuccinate (CHEMS), cholesterol (Chol), dioleoyl phosphatidyl ethanolamine (DOPE) and Egg yolk phosphatidyl choline (EPC) were purchased from Avanti Polar lipids (Alabaster, AL, USA). Plasmid DNA encoding GFP-luciferase fusion protein (pEGFPluc) was obtained from BD Bioscience Clontech (Palo Alto, CA USA). Rhodamine-labeled transferrin (Rh-Tf) and Sulforhodamine B (S-Rho) was purchased from Molecular Probes (Eugene, OR USA). STR-R8 was synthesized as described previously [18].

2.2. Quantification of cellular uptake of liposomes by flow cytometry

The cellular uptake of R8-liposomes and Rh-Tf was assessed by flow cytometry. Liposomes composed of EPC, Chol and STR-R8 (7:3:0.5 molar ratio) were prepared by the hydration method. A lipid film was produced by evaporation of a chloroform solution of 20 μmol lipids with a rotary evaporator under reduced pressure in a round-bottom flask to give a thin film. Hydration buffer (2 ml), PBS containing 1 mM of S-Rho was then added followed by vortexing to hydrate the lipid. Size was controlled by extrusion with a Mini-Extruder (Avanti Polar Lipids), through polycarbonate membrane filters (Nuclepore) with 400, 200 nm pore diameter (11 times each) and 100 nm pore diameter (21 times). Unencapsulated S-Rh was separated on a Bio-Gel A-1.5 m column (100–200 mesh) equilibrated with PBS. For the quantification of cellular uptake, NIH3T3 cells were seeded at a density of 1.0×10^5 cells per 6-well plate in growth media. After 24 h, the cells were pre-incubated for 30 min at 37 or 4 °C then incubated with R8 liposomes (0.1 mM) or Tf-TMR (tetramethyl rhodamine) (2 μM) in serum free medium for 1 h. Cell surface-bound R8-liposomes were removed as demonstrated previously with minor modifications [23]. Lundberg et al. showed that TAT-GFP, R8-GFP or K8-GFP protein conjugates bound to the cellular surface were nearly completely washed out by a single wash with PBS supplemented with heparin (5 unit/ml) [23]. However, this procedure was not sufficient to completely remove surface-bound R8-liposomes, presumably due to strong binding via its high density of R8-moiety (data not shown). To solve this problem, cells were washed 3 times with ice-cold PBS supplemented with a higher concentration of heparin (20 units/ml) for 3 times. Cellular binding of R8-liposomes encapsulating S-Rho was assessed by confocal laser scanning microscopy. After the incubation of cells with R8-liposomes at 37 °C for 1 h, cell-surface binding of R8-liposomes encapsulating S-Rho were clearly observed without a wash (Fig. 1A). In contrast, after the washing with PBS supplemented with heparin (20 units/ml), cell-surface binding of R8-liposomes were rarely observed as shown in Fig. 1B. Therefore, this condition is adequate for removing cell surface-bound R8-liposomes. For the analysis by flow cytometry, cells were once washed with PBS supplemented with heparin (20 units/ml), and then trypsinized and collected in an eppendorf tube, and then washed two additional times by repeating the precipitation of the cells by centrifugation (1500 rpm, 4 °C, 5 min) and resuspension in 1 ml of heparin-PBS. Finally, cells were suspended in 1 ml of PBS. The cell suspension was then filtered through a nylon mesh to remove cell aggregates and dust, and cells were analyzed with a flow cytometer (FACScan, Becton Dickinson).

2.3. Encapsulation of pDNA to the R8-modified liposome

For the quantification of cellular uptake and the nucleus-association of liposomes, plasmid DNA (pDNA) was encapsulated in R8-liposomes as described previously [32]. pDNA (0.1 mg/ml) was then condensed with poly-L-lysine by mixing equal volumes of these solutions at a charge ratio of 2.4 by vortexing at room temperature. After the condensation, a lipid film was produced by evaporation of a chloroform solution with 137.5 nmol of lipid (DOPE/CHEMS = 9:2 (molar ratio)) on the bottom of the glass tube. Then, 0.25 ml of the complex solution was added, followed by incubation for 10 min to hydrate the lipids. The final solution of the lipid was 0.55 mM. The hydrated solution was sonicated in a bath-type sonicator (125 W, Branson Ultrasonics, Danbury, CT) to complete the packaging. Finally, the surface of the liposomes was modified with R8 by incubation with a STR-R8 (5 mol% lipids) solution for 30 min at room temperature. Consistent with our previous report, we denote this pDNA-encapsulating R8-liposome as an R8-modified multifunctional envelope-type nano device (R8-MEND) [32].

2.4. Quantification of cellular uptake and nucleus—association of pDNA-encapsulating liposomes by TaqMan PCR

For the cellular uptake study, 2×10^5 cells were seeded on a 6-well cell culture plate for 24 h. The cell culture medium was then replaced with 1 ml of FCS-free media and the suspension was incubated for 30 min at 37 °C or 4 °C. The R8-MEND corresponding to 2 μg of pDNA (24 nmol of total lipid) was added, followed by an additional incubation for 1 h. As a control study, 2 μg of pDNA condensed with LipofectAMINE PLUS (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol was used. Cells were washed 3 times with PBS supplemented with heparin (20 units/ml) to remove cell surface-bound R8-MEND. To quantify the nuclear association of pDNA, the nuclear fraction was further purified. It is likely that cationic R8-MEND after binding on the cell surface may become redistributed to the outer surface of the nucleus during the purification procedure. To exclude this possibility, all of the purification

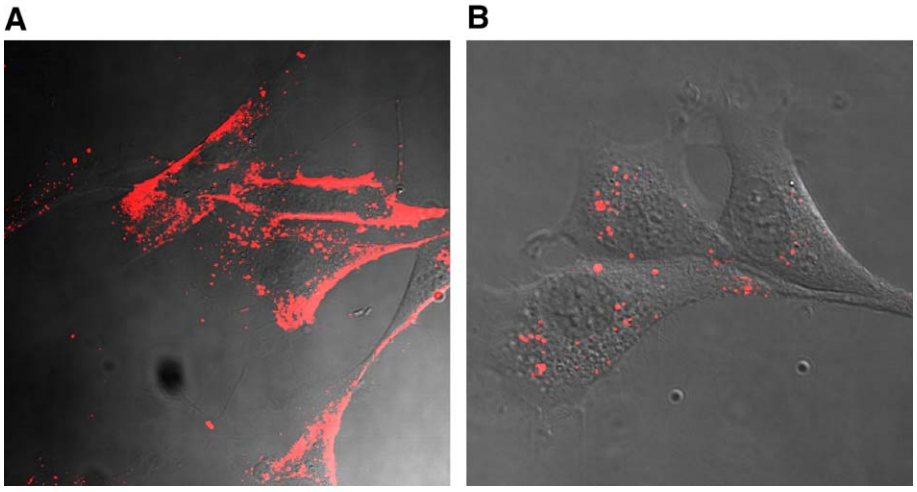

Fig. 1. Heparin-dependent removal of R8-liposomes from cellular surface. R8-liposomes encapsulating S-Rho were incubated for 1 h at 37 °C. Without washing (A), cellular binding of R8-liposomes was observed (arrowhead). After washing with ice-cold PBS supplemented with heparin (20 U/ml) for 3 times (B), cellular binging of

processes were performed in CellScrub buffer (Gene Therapy Systems Inc.), which was originally used for removing the cell surface-bound lipoplex. This reagent is capable of washing out the nuclear surface-bound R8-MEND, and to also interrupt the redistribution of the cytoplasmic R8-MEND to the nuclear fraction during the purification of the nuclei. In fact, after the incubation of R8-MEND and isolation of the nuclei in the presence of CellScrub buffer, the nuclear distribution was negligible (less than 0.1% of applied R8-MEND). The heparin-washed cells were suspended in 375 μl of CellScrub Buffer, and 125 μl of cell lysis solution (2% IGEPAL CA630, 40 mM NaCl, 12 mM $MgCl_2$ and 40 mM Tris–HCl, pH 7.4) was then added. The suspension was centrifuged at 9200×*g* for 2 min at 4 °C, and the supernatant was removed. This procedure was repeated 3 times. The final pellet was used as the nuclear fraction.

DNA was purified from cell lysates or an isolated nucleus by means of a GenElute Mammalian Genome DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO), and subjected to TaqMan PCR with ABI PRISM® 7700 Sequence Detection System (Applied Biosystems) as reported previously [33]. As a reference, a dilution series of pDNA3.1-GL3 was run along with the samples. The sequence of the probe was 5′-CCGCTGAATTGGAATCCATCTTGCTC-3′ with FAM as a fluorescent dye on the 5′ end and TAMRA as a fluorescence quencher dye at the 3′ end. This probe is designed to anneal to the target between the sense primer (5′-TTGACCGCCTGAAGTCTCTGA-3′) and the antisense primer (5′-ACACCTGCGTCGAAGATGTTG-3′) in the luciferase pDNA sequence. The number of β-actin DNA was also determined by the ABI PRISM® 7700 Sequence Detection System. PCR was performed according to the manufacturer's instructions with 0.5 μM each of the respective forward: 5′-TGCGTGACAT TAAGGAGAAGCTGTG-3′ and reverse: 5′-CAGCGGAACCGCTCATTGC CAATGG-3′ primers, and QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany). A linear relationship between the number of cells and the threshold cycle for the β-actin gene amplification was confirmed (data not shown). The amount of nucleus-associated pDNA was normalized by the number of nuclei quantified by the copy number of β-actin DNA.

2.5. Imaging of intracellular liposomes by confocal laser scanning microscopy

1×10^5 cells were seeded in 2 ml of cell culture medium on a 35 mm/GLASS BASE DISH (IWAKI, Chiba Japan) for 24 h. Plasma membranes were stained with PKH67 GREEN FLUORESCENT CELL LINKER (Sigma-Aldrich; St. Louis, MO USA) following to the protocol with some arrangements. After washing the cells were with serum-free medium, 100 μl of a PKH67 LINKER solution (10 μM) was added, and the cells were further incubated for 5 min at room temperature. The staining reaction was terminated by dilution of medium with 100 μl of serum-free medium. For the imaging of liposomes introduced at 37 °C and 4 °C, the cells were washed 3 times with 1 ml of PBS adjusted to the respective temperature. After washing, R8-liposomes encapsulating sulforhodamine (S-Rho) were immediately added, followed by incubation for 1 h at 37 °C and 4 °C, respectively. The cells were then washed with 1 ml of PBS containing heparin 3 times, and the cell culture medium was exchanged with 1 ml of Krebs-Henseleit Buffer. Confocal images were captured by a Zeiss Axiovert 2000 inverted fluorescence microscope equipped with a 63× NA 1.4 Planachromat objective (Carl Zeiss Co. Ltd., Jena, Germany). During the observation of cells, the temperature of the dish was rigorously controlled by means of a 0 °C∼65 °C thermo-control system (Carl Zeiss Co. Ltd.).

2.6. Assessment of transfection activity

For the transfection study, 4×10^4 cells were seeded on a 24-well plate and allowed to stand for 24 h. Before the transfection, the cell culture medium was replaced with 250 μl of serum-free medium, and then incubated for 37 °C or 4 °C for 30 min. R8-MEND corresponding to 0.4 μg pDNA was then added, followed by further incubation for 1 h at 37 °C or 4 °C. After the incubation, the cells were washed with 500 μl of PBS containing 20 units/ml of heparin sulfate 3 times, and heparin-free PBS 1 time. The cells were incubated with 1 ml of DMEM containing 10% fetal calf serum and incubated in a CO_2 incubator. At the indicated times, the cells were washed with PBS, and solubilized with reporter lysis buffer (Promega, Madison, WI). The luciferase activity was initiated by the addition of 50 μl of luciferase assay reagent (Promega) into 20 μl of the cell lysate, and measured by means of a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (PIERCE, Rockford, IL).

3. Results

3.1. Evaluation of the effect of temperature on the cellular uptake of R8-liposomes

The cellular uptake of R8-liposomes encapsulating S-Rho was investigated by flow cytometry at 37 °C and 4 °C. The size and ζ-potential of the prepared liposome are summarized in Table 1. Transferrin (Tf), a common marker of clathrin-dependent endocytosis, was used as a control to evaluate the inhibition of energy-dependent vesicular transport at 4 °C. Without washing the surface-bound liposomes, a comparable or higher degree of cellular association was observed after the incubation at 4 °C compared with that after the incubation at 37 °C (data not

Table 1
The size and ζ potential of prepared liposome

	Size (nm)	ζ potential (mV)
R8-liposome	103.3 ± 11.8	37.2 ± 6.8
R8-MEND	297.9 ± 41.3	39.5 ± 12.4

The hydrodynamic diameter was measured by quasi-elastic light scattering by means of an electrophoresis light scattering spectrophotometer.

shown). As shown in Fig. 1, the heparin wash efficiently removed the cell surface bound of R8-liposomes (see Materials and methods). Even after the removal of surface bound liposomes, a FACS analysis revealed that the internalization of R8-liposomes was only slightly inhibited at the low temperature used (Fig. 2A), while the internalization of Tf was significantly inhibited (Fig. 2B). The mean fluorescence intensity of 10,000 analyzed cells is shown in Fig. 2C. Approximately 80% of the cellular uptake of R8-liposomes was retained, even at 4 °C, while uptake of Tf was drastically decreased to approximately 30%.

To quantify cellular uptake and following nuclear delivery by TaqMan PCR, plasmid DNA was also encapsulated in the R8-liposome. Here, we denote this liposome as R8-MEND, consistent with a previous report [32]. Compared with R8-liposomes encapsulating S-Rho, the size of the R8-MEND was approximately 3-times larger (Table 1). The cellular uptake of the R8-MEND at low temperature was also confirmed (Fig. 3). As a control, pDNA condensed by LipofectAMINE PLUS, a commercially available cationic liposome was used, since it was previously reported that its internalization was severely inhibited at low temperature [19]. Consistent with the results obtained with the R8-liposome, a major portion (>50%) of the cellular uptake of R8-MEND was retained even by incubation at 4 °C, where the uptake of lipoplex was diminished to less than 30%.

3.2. Mechanism of uptake of R8-liposomes introduced at 37 °C and 4 °C

The involvement of vesicular transport in the cellular uptake of R8-liposomes was investigated by means of dual imaging of

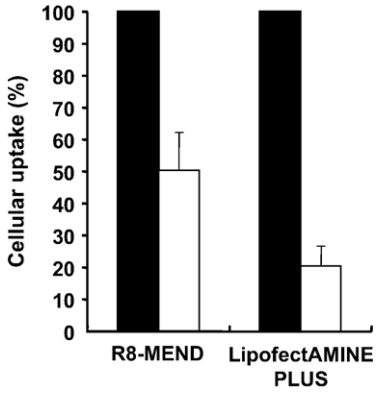

Fig. 3. Quantitative comparison of the uptake of R8-MEND introduced at 37 °C and 4 °C. NIH3T3 cells were incubated with the R8-MEND at 37 °C and 4 °C for 1 h to evaluate cellular uptake. Uptake was quantified in terms of the number of copies of luciferase genes by TaqMan-PCR. Closed and open bar represents cellular uptake of pDNA quantified by TaqMan PCR after the incubation at 37 °C and 4 °C, respectively. Cellular association was normalized by the number of cells, which is quantified by the number of copies for genomic β-actin gene.

an R8-liposome encapsulating S-Rho and PKH67-labeled plasma membrane. After incubation at 37 °C for 1 h, the major fraction of the R8-liposomes was co-localized with intracellular vesicular structures, suggesting that R8-liposomes are mainly taken up to the cells by vesicular transport (Fig. 4A). Some liposomes were detected as red clusters close to the nucleus, suggesting that liposomes were partially released from the vesicular compartment. When incubated at 4 °C, the fluorescence signals of PKH67 were dominantly detected on the surface of the cells, suggesting that the vesicular transport system was severely interrupted under these conditions (Fig. 4B), whereas a small number of green clusters were still observed just beneath the plasma membrane. Even under these conditions, the fluorescence signal of S-Rho was detected in the intracellular region. Magnified images further reveled that intracellular signals corresponding to S-Rho (indicated as red arrowheads in Fig. 4B; inset 1) were detected close to the green signals derived from the PKH67 signal (indicated as green arrowheads in Fig. 4B; inset 1), but were not colocalized with each other. In this case,

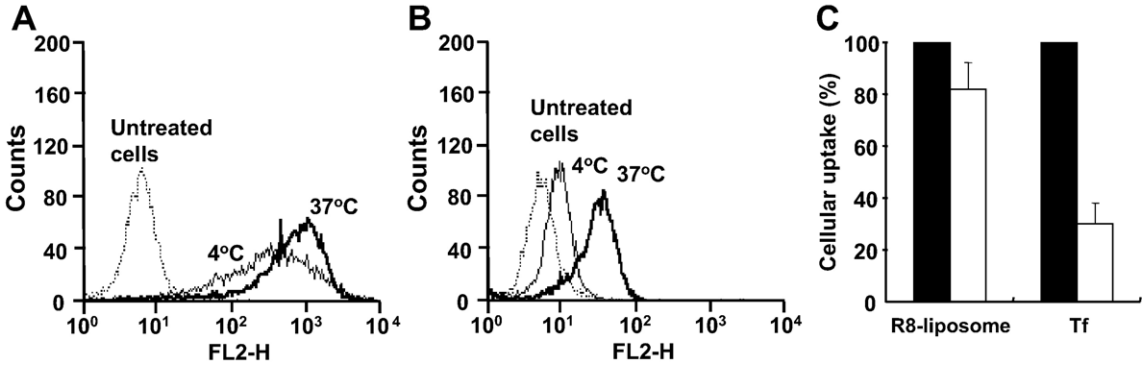

Fig. 2. Quantitative comparison of the uptake of R8-liposomes introduced at 37 °C and 4 °C. R8-liposomes encapsulating S-Rho (A) and Rho-Tf (B) were incubated with NIH3T3 cells in serum free medium for 1 h. After washing with 20 U/ml of heparin, the cells were detached by treatment with a trypsin solution. Cells were collected by centrifugation, and resuspended in 100 μl of PBS. The cells were analyzed with a flow cytometer, and the mean fluorescence was then plotted (C). Thick and thin lines represent the cellular uptake of R8-liposomes introduced at 37 °C and 4 °C (A and B). Hatched lines represent the non-treated control. (C) Closed and open bar represents mean fluorescence of analyzed 10000 cells after the incubation at 37 °C and 4 °C, respectively.

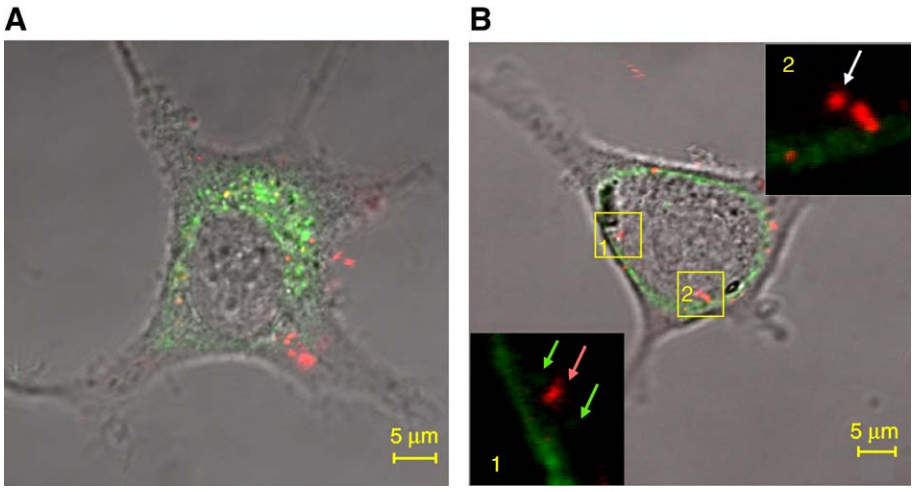

Fig. 4. Dual imaging of the R8-liposome encapsulating S-Rho and plasma membrane labeled with PKH67. After staining the plasma membrane with PKH67, R8-liposomes encapsulating S-Rho were immediately added, followed by incubation for 1 h at 37 °C (A) and 4 °C (B), respectively. Fluorescence images were captured by means of confocal laser scanning microscopy. During the observation of images, the temperature of the dish was rigorously controlled by 37 °C or 4 °C by a thermo-control system. The white and red arrowhead in (B; inset 1 and 2, respectively) indicates intracellular R8-liposomes taken up at 4 °C. Green arrowhead in inset 2 represented intracellular PKH67 signals.

R8-liposomes may be taken up via temperature-independent invagination, followed by their rapid escape to the cytosol. Moreover, some red signals were far away from the green signals (Fig. 4B; inset 2). This indicates that the R8-liposomes were alternatively internalized via vesicular transport-independent pathway. Confocal images also revealed that R8-liposomes were localized close to the plasma membrane (Fig. 3B: arrowhead), but were not localized on peri-nuclear region.

3.3. Nuclear association and trans-gene expression of R8-MEND introduced at 37 °C and 4 °C

The nuclear association of R8-MEND after the internalization at 37 °C and 4 °C (denoted as R8-MEND$_{37}$ and R8-MEND$_4$) was quantified by means of TaqMan PCR (Fig. 5A). After the incubation of R8-MEND for 1 h at 37 °C and 4 °C, the cells were washed with a heparin solution to remove cell surface-bound R8-MEND, and further incubated at 37 °C for the indicated times. The nuclei were fractionated, and nucleus-associated pDNA was subsequently quantified by TaqMan PCR. A peak for the nuclear association of R8-MEND$_{37}$ appeared within 3 h, while the peak for R8-MEND$_4$ was observed at 6 h. These data indicate that the R8-MEND$_{37}$ reached the nucleus more rapidly than R8-MEND$_4$. The percent of nucleus-associated pDNA to the totally internalized one (F_{nuc}) is summarized in Table 2. Comparing the F_{nuc} at the time of peaking (at 3 h for the R8-MEND$_{37}$ versus at 6 h for the R8-MEND$_4$), the R8-MEND$_{37}$ showed an approximately 4.6 times higher value than that of R8-MEND$_4$, (24.8% vs. 5.43%). Collectively, R8-MEND$_{37}$ was able to gain access to the nucleus more rapidly and more efficiently compared with the R8-MEND$_4$.

To compare the transgene expression between R8-MEND$_{37}$ and R8-MEND$_4$, cells were incubated at 37 °C for the indicated

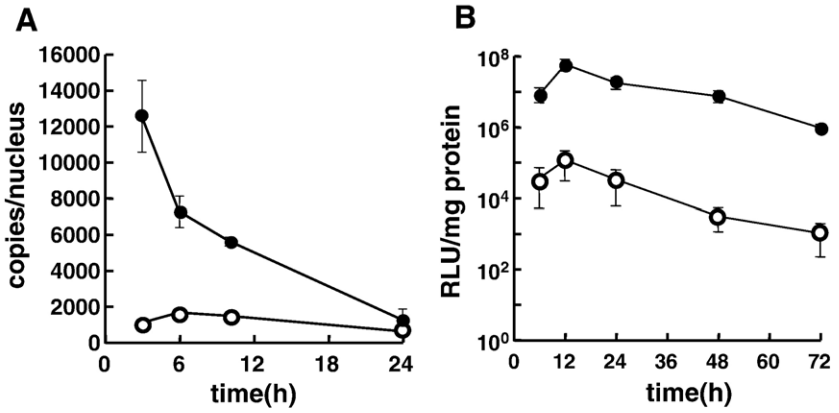

Fig. 5. Time profiles for the nuclear associating pDNA and transfection activity after the introduction of R8-MEND at 37 °C and 4 °C. (A) After the introduction of R8-MEND at 37 °C and 4 °C, cells were washed with heparin to remove the membrane-bound R8-MEND. Nucleus-associated pDNA was quantified by nuclear fractionation followed by TaqMan PCR. The number of the nucleus-associated pDNA was normalized by the number of nucleus quantified by the copy number of β-actin genome. (B) Transfection activity of R8-MEND introduced at 37 °C and 4 °C at the indicated times. The results obtained for the incubation at 37 °C and 4 °C are represented as closed and open circle, respectively. The vertical axis represents luciferase activity expressed as relative light units (RLU)/mg protein.

times after washing with a heparin sulfate (Fig. 5B). At all the indicated times, the transgene-expression of the R8-MEND$_{37}$ was 2- or 3- orders of magnitude higher than that for the R8-MEND$_4$. It should be noted that the transfection activity of the R8-MEND incubated at 37 °C and 4 °C was comparable, when the cell surface-bound R8-MEND was not removed by heparin sulfate treatment (data not shown). This strongly suggests that the intracellular trafficking of R8-MEND introduced in the cells at 4 °C was distinct from that of the plasma membrane-bound R8-MEND after cell culture medium was warmed from 4 °C to 37 °C. The amount of protein in the wells showed quite comparable values between R8-MEND$_{37}$ and R8-MEND$_4$ (data not shown). Therefore, the prominent difference in transgene expression cannot be accounted for by cytotoxic effects during the incubation at 4 °C. Finally, "transgene expression normalized by nuclear pDNA" was compared at two time points (Table 3) [34]. At 6 h, R8-MEND$_{37}$ exhibited dramatically higher values (51-fold) compared with that of R8-MEND$_4$. Moreover, at 24 h, the difference was greater (247-fold). These data suggest that the intrinsic activity of pDNA to produce a gene product was significantly impaired, when introduced by R8-MEND$_4$ compared with that introduced by R8-MEND$_{37}$.

4. Discussion

Recent studies have indicated that vesicular transport plays an important role in the cellular uptake of PTD-modified cargos [10,14,17,22–29]. Inhibitors and/or biomarkers for various vesicular transport systems were used in attempt to identify the pathway responsible for cellular uptake. These studies revealed that clathrin-mediated endocytosis [22,23,30], macropinocytosis [14,17,29,30] and caveolae-mediated endocytosis [26] are all involved in the uptake of PTD peptides ser se, or PTD-modified proteins, while major determining factor in sorting the internalization pathway remains obscure. Vesicular transports are driven by coupling with ATP hydrolysis. Therefore, these pathways should be inhibited at low temperatures. In fact, the cellular uptake of transferrin was drastically inhibited to approximately 30%. In the present study, the majority of R8-liposomes were taken up by cells, even at 4 °C. Initial studies indicated that PTD is able to penetrate the cells via endocytosis-independent pathways since cellular uptake was unaffected by low-temperature incubation and with endocytosis inhibitors [13]. Whereas these results were artifacts resulting from cell fixation [22], recent studies using living cells still support the internalization of a certain kind of PTD even at the low temperature

Table 2
Efficiency of nuclear association of R8-MEND37 and R8-MEND to the totally internalized MENDS

	Uptake (copies/cell)	Percent of nuclear association of pDNA to the total cellular uptake			
		3 h	6 h	10 h	24 h
37 °C	5.05×10^4	24.8	14.3	11.1	2.40
4 °C	2.96×10^4	3.53	5.43	4.82	1.91

The efficiency of nuclear association was represented as a percent of nuclear association of pDNA to the total cellular uptake.

Table 3
Comparison of the transfection efficiency normalized by nuclear pDNA transfected by R8-MEND$_{37}$ and R8-MEND$_4$

	Time post-injection (h)	
	6 h	24 h
37 °C	1.18×10^3	1.45×10^4
4 °C	23.1	58.5

Transfection activity and nuclear pDNA at 6 h and 24 h was based on Fig. 5A and B, respectively. Values are expressed as RLU/mg protein/copy.

[17,31]. Nakase et al., quantitatively compared the cellular uptake of R8 by the incubation at 37 °C and 4 °C, and found that approximately 60–70% of the cellular uptake of R8 was retained on incubation at 4 °C [17]. This is consistent with our findings showing that approximately 80% and 50% of the cellular uptake of R8-liposomes and R8-MEND was retained, on incubation at 4 °C (Figs. 2 and 3).

In contrast to the mechanism for cellular uptake at 37 °C, very little information is available concerning the uptake at 4 °C. The involvement of non-vesicular transport in the uptake of R8-liposomes was further investigated by the dual labeling of plasma membranes with PKH67 and S-Rho encapsulated in the liposomes (Fig. 3). Since PKH67 stably incorporates a fluorescent dye into the lipid regions of cell membranes with its long aliphatic tail, all of the internalized vehicles after the membrane staining should be labeled irrespective of the internalization pathway. In this observation, the temperature of the dish was carefully kept by means of a thermo-control system, and cell surface-bound R8-liposomes were removed by treatment with a heparin sulfate solution in order to exclude the possibility that cell-surface bound R8-liposomes during the incubation at 4 °C were internalized via an energy-dependent vesicular transport system by temporal and local warming caused by laser irradiation. As a result, internalization of plasma membranes labeled with PKH67 was rarely observed at 4 °C (Fig. 4B). Even in this condition, the internalization of R8-liposome was observed in the cytoplasm, suggesting that the R8-liposome probably penetrated into the cells even at 4 °C. Furthermore, the localization of R8-liposomes were restricted to a specific areas in close proximity to plasma membrane (Fig. 4B: insets 1 and 2), as was also observed for the R8 peptide [17]. This suggested that migration of R8-liposomes taken up at low temperature was severely restricted.

It has been proposed that guanidinium-rich peptides interact with negatively charged, bidentate hydrogen bond acceptor groups of endogenous membrane constituents, forming a membrane-soluble ion pair complex, which then migrates into the lipid bilayer [35]. However, this hypothesis was only applied to small molecules (MW ca. < 3000). An alternative pathway such as temperature-insensitive potocytosis [36] may be responsible for the translocation of R8-liposomes after the formation of ion pairs. The temperature-insensitive invagination is supported by the fact that some PKH-67 signals were observed in the intracellular region, but were restricted to directly beneath the plasma membrane (indicated as green arrowheads in Fig. 4B; inset 1). Furthermore, a part of intracellular R8-liposomes were detected close to the PKH-67 signal (indicated as red arrowhead in Fig. 4B; inset 1). These findings suggest that R8-liposomes

were partially taken up by temperature-insensitive potocytosis, followed by rapid release to the cytosol. In addition, some R8 liposomes were detected in the intracellular region away from the PKH67 signal (indicated as white arrowhead in Fig. 4B; inset 2), suggesting that alternative mechanism such as penetration may also play a role. The cellular uptake of R8-MEND was more temperature-sensitive compared to the R8-liposome (50% vs. 20% inhibition) as shown in Figs. 2C and 3. Since the mean diameter of the R8-MEND was significantly larger than that of R8-liposomes (Table 1), smaller sized of macromolecules may be more acceptable to these temperature-independent cellular uptake mechanisms. An investigation of molecular mechanism for the cellular uptake is ongoing in our laboratory.

In the 37 °C incubation, R8-liposomes, free from the co-localization with plasma membranes, were observed at the perinuclear region, which is distinct from the localization of R8-liposomes incubated at 4 °C. These liposomes could then be transported to the perinuclear region by intracellular sorting by vesicular trafficking, and then released to the cytosol. Some recent studies indicated that the endosomal escape of PTD is inhibited by ammonium chloride [25] or chroloquine [37]. Thus, it is possible that the endosomal escape of R8-liposomes is coupled with acidification in the vesicular compartment. In this sense, clathrin-mediated endocytosis [22] or macropinocytosis [23] is a more plausible uptake pathway for the R8-liposomes compared with caveolae-mediated endocytosis [26], since the pH in caveolae does not decrease after invagination. In fact, we have demonstrated that cellular uptake R8-liposomes used in the present study were inhibited by amiloride, which is a inhibitor of the macropinocytosis [30].

Considering the relationship between intracellular fate and time profiles for nuclear association, the findings strongly suggest that the involvement of vesicular transport to the peri-nuclear region is advantageous for the rapid and efficient nuclear delivery of R8-MEND introduced at 37 °C (Fig. 5A and Table 2). As mentioned above, R8-MEND$_{37}$ appears to be released from vesicular compartments that are close to the nucleus after active vesicular transport, while R8-MEND$_4$ appears to be directly introduced to the cytoplasm in close proximity to the plasma membrane. Therefore, R8-MEND$_4$ needs to travel longer distance in the cytoplasm to reach the nucleus compared with the R8-MEND$_{37}$. It has been reported that the movement of macromolecules in the cytoplasm is severely restricted [38]. If this is true, then, the speed and efficiency of R8-liposomes for reaching the nucleus is closely related to the distance from the cytoplasmic R8-liposome to the nucleus. In fact, Daum et al., indicated that the trans-gene expression of pDNA microinjected into the cytoplasm far from the nucleus was much lower than that injected close to the nucleus.

Finally, time profiles for the transgene expression were evaluated. At 37 °C, transgene expression was peaked at 12 h and then decreased slightly (approximately 30% of the peak luciferase activity) at 24 h, which is inconsistent with the fact that nuclear pDNA peaked at 3 h and then decreased monotonically. The synthesis of proteins is a product of the nuclear transcription of pDNA and subsequent translation. As a result, time lag between peak of nuclear pDNA (within 3 h) and luciferase activity (12 h) may be derived from the time required for the nuclear transcription and translation. Concerning the elimination process, a decrease in transgene expression from 12 h to 24 h was less prominent than that of nuclear pDNA. Since the apparent luciferase activity is balanced by synthesis and clearance of gene products, the inefficient clearance of a gene product may reflect the moderate luciferase activity, even though biosynthesis may be reduced in response to the time-dependent decrease in nuclear pDNA. In this sense, "transgene expression normalized by nuclear pDNA" calculated as the trans-gene expression divided by the gene copies in the nucleus (Table 3) is not necessarily a direct index of intrinsic activity of pDNA for the biosynthesis. However, if the intrinsic activity of pDNA to produce the gene product was comparable between R8-MEND$_{37}$ and R8-MEND$_4$, "transgene expression normalized by nuclear pDNA" (Table 3) may represent comparable values based on the hypothesis that the clearance of a gene product should be comparable regardless of the conditions used for the introducing the R8-MEND. R8-MEND$_{37}$ exhibited approximately a 500 times higher trans-gene expression compared with R8-MEND$_4$ (Fig. 5B). In comparing "transgene expression normalized by nuclear pDNA", R8-MEND$_{37}$ shows 2- or 3 orders of magnitude higher values than those of R8-MEND$_4$ (Table 3). As mentioned in Materials and methods, the core particle of pDNA condensed with polycations was coated with a lipid envelope (DOPE/CHEMS, a pH sensitive fusogenic lipid composition [39,40]) to form the MEND structure [32]. It has been demonstrated that trans-gene expression after the nuclear microinjection of pDNA is strongly impaired when it is injected in the form of a lipoplex [41,42]. suggesting that pDNA should be free from cationic lipid components so as to be efficiently recognized by intra-nuclear transcription factors. When R8-MEND$_{37}$ escapes from the vesicular compartment, the lipid envelope is presumably consumed by fusion with the cellular membrane, forming a hexagonal structure-II structure in response to the low intra-vesicular pH [43]. As a result, pDNA encapsulated in an R8-MEND$_{37}$ may be more prominently subject to the transcription process compared with that in the R8-MEND$_4$.

Collectively, it was verified that R8-liposomes, when incubated at a temperature of 4 °C, enter cells via a unique pathway distinct from that introduced at 37 °C, whereas subsequent nuclear delivery was less efficient compared with that of R8-liposomes introduced at a temperature of 37 °C. This conclusion provides encouragement for using a strategy involving the endogenous vesicular sorting system for the nuclear targeting of macromolecules.

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