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**Topology of octaarginines (R8) or IRQ ligand on liposomes affect the contribution of
macropinocytosis- and caveolae-mediated cellular uptake**

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ABSTRACT

It was recently reported that liposomes modified with octaarginine (R8) and its analogue peptide (IRQRRRR: IRQ) are taken into NIH3T3 cells by unique pathways, macropinocytosis and caveolae-mediated endocytosis, respectively. This study evaluated the topology of these peptides as it relates to the uptake routes of liposomes, where they are modified either directly on the surface, or on the edge of a polyethylene glycol (PEG) spacer. The uptake mechanism of peptide-modified liposomes and peptide-modified PEG-liposomes was investigated by confocal laser scanning microscopy. To determine the contribution of clathrin-mediated endocytosis, macropinocytosis and caveolar endocytosis to the uptake of liposomes, the uptake was evaluated in the presence of these specific inhibitors. The uptake pathway changed from macropinocytosis to clathrin-mediated endocytosis when R8 was modified on the edge of a PEG spacer, indicating that the flexible display of R8 impaired the induction of macropinocytosis. However, the contribution of caveolae-mediated endocytosis increased when IRQ was conjugated to the distal end of the PEG chain, suggesting that flexible surface display enhanced IRQ recognition by the specific molecules in the caveolae. The present results demonstrate that topology control of the ligand affects the contribution of the entry pathway, depending on the uptake mechanism.

KEY WORDS: R8, IRQ, topology control, clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis

1. Introduction

Protein transduction domains (PTDs), small basic peptides containing several arginine residues, have recently been used for the cellular delivery of biologically active macromolecules, including proteins, peptides, nucleic acids and liposomes [1-3]. One example is the human immunodeficiency virus (HIV) Tat-derived peptide. Although modification of Tat peptide enhanced cellular uptake of various macromolecules, its uptake mechanism remains to be elucidated [4-6]. It is likely that the cellular uptake pathway of Tat-modified macromolecules is affected by various factors such as the type of peptide, the nature of the cargo, and the connecting linker [7].

Based on the high arginine content in the Tat sequence, Futaki et al. synthesized a polypeptide composed solely of arginine residues [8], which can deliver macromolecules as efficiently as a Tat peptide [9,10]. Stearylated octaarginine (STR-R8) is a multifunctional device. First, it can condense DNA, and can deliver it to cells for significant gene expression [9]. Presumably due to the synergistic effect of hydrophobic and hydrophilic components in one molecule, it is useful for the condensation of small interference RNA (siRNA) [10]. Its other key function is to serve as cellular uptake inducers for liposomes. Because of its stearyl moiety, it can easily incorporate into the lipid bilayer, and results in a surface display of R8 on the liposomes (R8-Lip). Furthermore, it has been demonstrated that the uptake of R8-Lip changes depending on the peptide density. Liposomes modified with STR-R8 at low density (0.8 mol% of total lipid) were internalized via clathrin-mediated endocytosis, while liposomes modified at high density (5 mol% of total lipid) were internalized via macropinocytosis [11]. Since the macropinocytosis pathway is advantageous for lysosomal degradation, R8-Lip is highly efficient for the delivery of plasmid DNA, siRNA and proteins. Meanwhile, a novel ligand peptide that is also rich in arginine residues, IRQRRRR (IRQ) by *in vivo* phage display, was identified [12]. IRQ can

modify liposomes by incorporating stearylated IRQ in the lipid bilayer. In contrast to R8-Lip, the IRQ-modified liposomes (IRQ-Lip) use unique pathways, caveolar endocytosis and clathrin-mediated endocytosis [12]. These results indicate that not only the density of the peptide, but also minor substitution of peptide sequences, may affect the entry mechanism of arginine-rich peptides.

Another possible factor that affects cellular uptake is the flexibility of the ligand. A previous study showed that mobile motion of maltose-conjugated α -cyclodextrins in the polyrotaxane structure contributes to the improvement of an affinity to concanalin A [13]. Other studies showed that the attachment of Tf to the surface of liposomes with a PEG spacer (Tf-PEG-Lip) enhances cellular uptake efficiency and speed, compared with liposomes modified with Tf, albeit without the PEG spacer [14]. However, the effect of the topology of the ligand on the internalization mechanism is still unknown in arginine-rich peptides. This study investigated the cellular uptake pathway of liposomes modified with R8 and IRQ, either directly on the liposomes (R8-Lip and IRQ-Lip, respectively), or on the edge of the polyethylene glycol (PEG) spacer (R8-PEG₂₀₀₀-Lip and IRQ-PEG₂₀₀₀-Lip, respectively).

2. Materials and methods

2.1 Materials

Stearylated-octaarginine (STR-R8) was synthesized as described previously [15]. Synthesis of STR-IRQ was performed following the procedure used for the synthesis of STR-R8, in which the stearyl moiety was attached to the N-terminal of the IRQ peptide. R8-PEG₂₀₀₀-DSPE and IRQ-PEG₂₀₀₀-DSPE were synthesized using a single-step reaction of Mal-PEG₂₀₀₀-DSPE with either Cys-R8 or Cys-IRQ peptide, following the procedure used for the synthesis of demorphin-PEG-DSPE [16].

2.2 Preparation of liposomes

Four types of liposomes, R8-Lip, IRQ-Lip, R8-PEG₂₀₀₀-Lip and IRQ-PEG₂₀₀₀-Lip, were prepared using the hydration method, and were composed of Cho and EPC in a molar ratio of 3:7 plus additional STR-R8, STR-IRQ, R8-PEG₂₀₀₀-DSPE, or IRQ-PEG₂₀₀₀-DSPE, at 5 mol% of total lipids. To label the R8-Lip and IRQ-Lip, Rh-DOPE (1 mol% of total lipid) was also added in lipid composition. Meanwhile, R8-PEG₂₀₀₀-Lip and IRQ-PEG₂₀₀₀-Lip were labeled by encapsulating rhodamine as an aqueous phase marker. The glass tube was then sonicated for approximately 1 min in a bath-type sonicator (125 W, Branson Ultrasonics, Danbury, CT). Liposomes were purified by gel filtration on a Sephadex G-100 1.5 column. The size distribution and zeta-potential of each sample were determined using a Zetasizer Nano ZS ZEN3600 (MALVERN Instrument, Worcestershire, UK).

2.3 The cellular uptake study by confocal laser scanning microscopy (CLSM)

To investigate the internalization mechanism of liposomes, 2×10^5 of NIH3T3 cells were seeded on a 35-mm glass-base dish (Iwaki, Chiba, Japan) in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 24 h. Before transfection, the cells were washed once with 1 ml of PBS and were pre-incubated with serum-free medium in the presence or absence of sucrose (0.4 M) for 30 min, amiloride (5 mM) for 10 min, and filipin (1 mg/ml) for 1 h. Liposomes corresponding to 0.050 mmol of total lipids were added to the cells and incubated for 1 h at 37 °C in the presence or absence of inhibitors. The cells were washed 3 times with 1 ml of ice-cold phosphate buffer saline (PBS) supplemented with heparin (20 units/ml) to remove surface-bound liposomes, as reported previously [11]. Finally, the cells were washed once with 1 ml of Krebs Henseleit buffer and observed by CLSM. Nuclei were stained with Syto-24 (final concentration 0.5 mM) for 20 min before microscopic analysis.

3. Results and discussion

An understanding of the cellular uptake mechanism is essential, since it is closely related to the subsequent intracellular trafficking and the function of carriers. In the present study, the cellular uptake of R8 and the IRQ-modified liposomes was evaluated in the presence of inhibitors.

The size and zeta-potential of liposomes were determined and are summarized in Table 1. Direct modification of R8 or IRQ to the liposomal surface rendered highly positively charged liposomes. The R8-Lip and IRQ-Lip exhibited zeta potential of approximately 38 and 35 mV, respectively. When DSPE-PEG was incorporated into the liposomes, negatively charged particles were formed (-32.5 mV). However, preparation of R8-PEG-Lip and IRQ-PEG-Lip neutralized the charge of approximately 1.4 – 2.5 mV, suggesting that these cationic peptides were actually attached to the edge of the PEG.

To identify the contribution of various endocytic pathways, the uptake of R8-Lip, IRQ-Lip, R8-PEG₂₀₀₀-Lip and IRQ-PEG₂₀₀₀-Lip was evaluated in the presence of inhibitors for macropinocytosis (amiloride) [17], clathrin-mediated endocytosis (sucrose) [18] and caveolar endocytosis (filipin III) [19]. The uptakes were determined using CLSM and after removing the surface-bound liposomes with a heparin wash. The uptake of high-density R8-Lip was inhibited by amiloride (Fig. 1B), whereas the use of a hypertonic medium (Fig. 1C) and the caveolar inhibitor filipin III (Fig. 1D) did not inhibit the uptake of R8-Lip. These results are consistent with the previous ones—the uptake of R8-Lip occurred mainly via macropinocytosis [11]. In contrast, the uptake of R8-PEG₂₀₀₀-Lip was not inhibited by amiloride and filipin III (Fig. 2B and 2D), but was strongly inhibited by a hypertonic medium (Fig. 2C). These results clearly indicate that R8-PEG₂₀₀₀-Lip is mainly taken up via clathrin-mediated endocytosis. Collectively, R8 modification on the edge of the flexible PEG moiety altered the cellular uptake pathway from macropinocytosis to classical

endocytosis.

It has been reported that glucosaminoglycan- and heparan sulfate-deficient CHO cells lack cellular uptake of R8 and Tat, suggesting that the interaction of membrane-associated proteoglycans and PTDs is essential to trigger the macropinocytosis-dependent uptake pathway [20]. Therefore, it is postulated that the binding of R8-Lip to the membrane-associating proteoglycans is essential for cellular uptake via macropinocytosis. As previously reported [21], liposomes modified with a high R8 density may strongly bind to the cell surface through ionic interactions with the cell-surface HSPGs. This strong and multiple binding in a localized area may promote proteoglycans (PGs) multimerization. The clustering of PGs then initiates phosphorylation of the cytoplasmic domains, which leads to a significant rearrangement in the cytoskeleton that stimulates macropinocytosis. Clustering of the PGs under the influence of cationic particles and cytoskeleton rearrangement in the presence of R8 were previously reported [7,22]. The authors hypothesized that the ability of the high R8 density to induce cytoskeleton rearrangement is decreased when R8 is attached to the distal end of the PEG chain. A flexible PEG spacer likely decreases the driving force to cluster the PGs, which results in an insufficient stimulation of ruffle formation. The classical endocytosis pathway then makes the main contribution to cellular uptake.

Meanwhile, IRQ resulted in a unique internalization mechanism of IRQ-modified liposomes through non-classical endocytosis. The uptake of IRQ-Lip was not inhibited by amiloride (Fig. 1F), suggesting that the uptake of IRQ-Lip involves a different uptake pathway, whereas IRQ also possessed multiple arginine residues. In contrast, the use of a hypertonic medium partially inhibited the uptake of IRQ-Lip (Fig. 1G). Furthermore, the use of filipin III strongly inhibited the uptake of IRQ-Lip (Fig. 1H). These results are in agreement with previous results—the internalization of IRQ-Lip occurs via clathrin-mediated endocytosis in parallel with caveolar endocytosis [12]. Basically, the

positive charges of liposomes trigger internalization into the cells due to the negative charges of cell surfaces. However, it is also probable that size is an important determinant of the mechanism by which particles enter cells. Evidently, the actual size of caveolae is very small (~70 nm) [23] for accommodating an IRQ-modified carrier, which may be as large as 200 nm. The results of the present study are consistent with prior studies showing that large latex beads preferentially enter via caveolar endocytosis, although the mechanism is unclear [24,25]. However, other studies showed that particles with sizes exceeding 200 nm were internalized via macropinocytosis rather than caveolar endocytosis [26]. Taken together, these results suggest that the preferred cellular uptake pathway of IRQ-Lip particles could not be simply discussed by its physicochemical properties such as charge and size. It is possible that a specific receptor on caveolae recognized the IRQ sequence on the liposomes and pulled it into the caveosome, and that some of the liposomes, outside of the recognition by specific receptors, were taken up via clathrin-mediated endocytosis by default.

The uptake of IRQ-PEG₂₀₀₀-Lip was not inhibited by amiloride and hypertonic medium (Fig. 2F-G), whereas the use of filipin III strongly inhibited the uptake of IRQ-PEG₂₀₀₀-Lip (Fig. 2H). These results clearly reveal that IRQ-PEG₂₀₀₀-Lip uses caveolar endocytosis as the major entrance route into cells. In this study, the size of IRQ-Lip and IRQ-PEG₂₀₀₀-Lip was 165-173 nm, larger than the reported size of the caveosome (~70 nm) [23]. Since large latex beads, approximately 500 nm in size, preferentially enter cells via caveolar endocytosis [24], suggesting that particle size is not a limiting factor for cellular entry via caveolae-mediated endocytosis. Recognition of target molecules in caveolae by the IRQ is possibly more important than the size of the particle itself, whereas the cell surface receptors mediating caveolar endocytosis of IRQ-modified nano carriers has yet to be identified. In addition, it is noteworthy that IRQ-PEG₂₀₀₀-Lip is

taken up into cells more specifically via the filipin III-sensitive pathway. Modification of IRQ ligand on the edge of the PEG moiety probably facilitates the multivalent binding of IRQ to the receptor and results in the increase of caveolae specificity. Since it is currently postulated that caveolae-mediated transcytosis is linked to transcytosis [23,27], IRQ-modified carriers would have the potential to traverse endothelial cells from the apical to the basal side to deliver macromolecules.

4. Conclusions

Modification of R8 on the edge of the PEG chain reduced the contribution of macropinocytosis-dependent uptake, whereas modification of IRQ with a PEG spacer increased the contribution of the caveolae-dependent pathway. These results collectively indicate that adequate topology control of the ligand is an important factor in the targeting of unique cellular uptake pathways, depending on the route and its mechanism.

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Figure Legends

Figure 1 Uptake of R8-Lip and IRQ-Lip in the presence of different specific inhibitors.

Confocal microscopic images of rhodamine-labeled R8-Lip and IRQ-Lip in NIH3T3 cells were captured after 1 h incubation in the absence (control) and the presence of amiloride, hypertonic medium, and filipin III. The nucleus was stained with Syto-24.

Figure 2 Uptake of R8-PEG₂₀₀₀-Lip and IRQ-PEG₂₀₀₀-Lip in the presence of different specific inhibitors.

Confocal microscopic images of rhodamine-labeled R8-PEG₂₀₀₀-Lip and IRQ-PEG₂₀₀₀-Lip in NIH3T3 cells were captured after 1 h incubation in the absence (control) and the presence of amiloride, hypertonic medium, and filipin III. The nucleus was stained with Syto-24.

TABLES

Table 1 The size and zeta-potential of prepared liposomes

Types of liposomes	Size (nm)	Zeta-potential (mV)
R8-Lip	185.0 ± 3.6	37.5 ± 3.5
IRQ-Lip	172.5 ± 4.9	35.0 ± 3.2
PEG ₂₀₀₀ -Lip	91.7 ± 2.6	-32.5 ± 4.3
R8-PEG ₂₀₀₀ -Lip	137.7 ± 6.5	2.5 ± 1.9
IRQ-PEG ₂₀₀₀ -Lip	164.7 ± 3.5	1.4 ± 0.8

Data represent means and SD of three different determinations

Figure 1

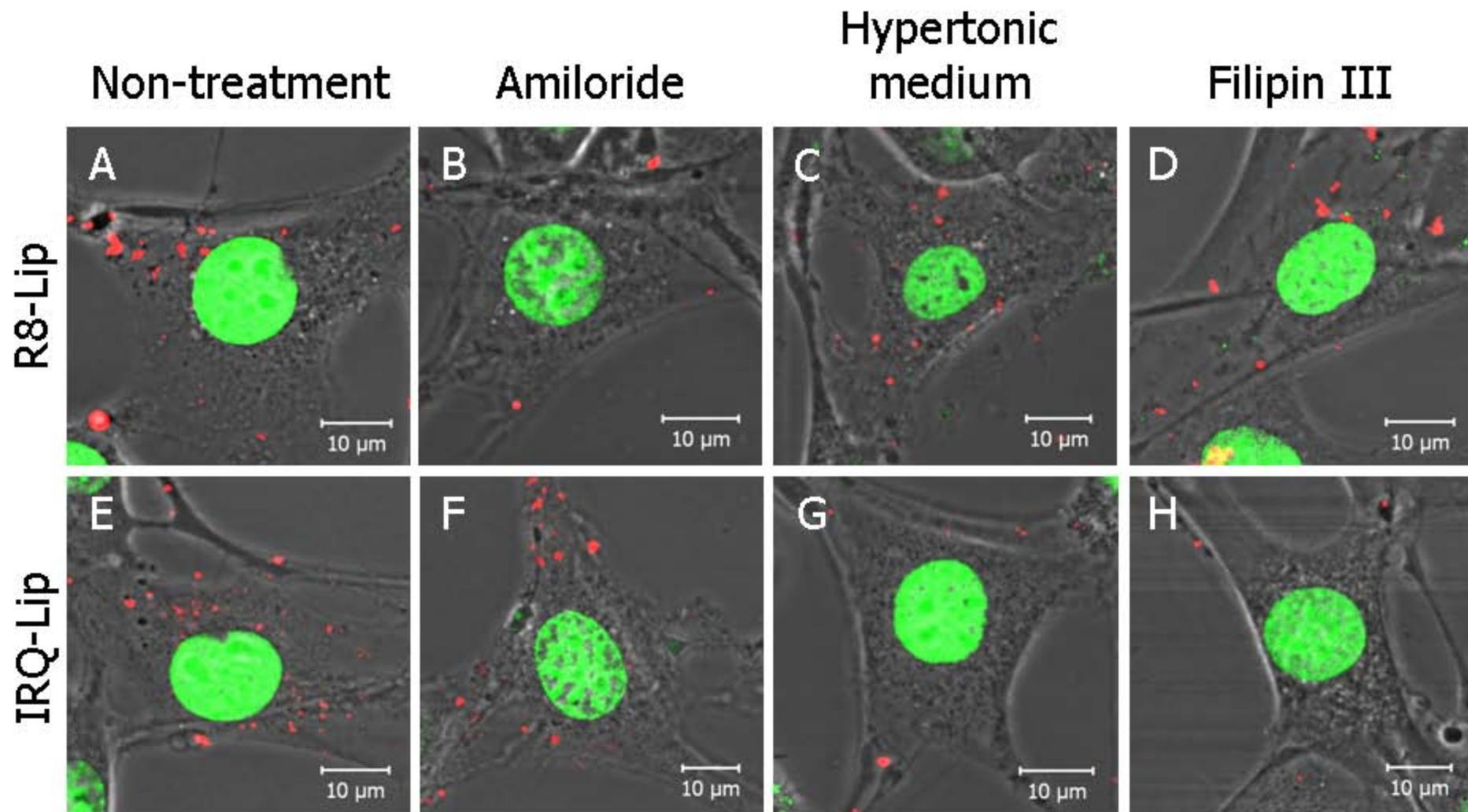


Figure 2

