Reactive & Functional Polymers 71 (2011) 340–343

Contents lists available at ScienceDirect

Reactive & Functional Polymers

journal homepage: www.elsevier.com/locate/react

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

Diky Mudhakir [1,2], Hidetaka Akita [2], Hideyoshi Harashima *

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan
Core Research for Evolution of Science and Technology (CREST), Japan Science and Technology Corporation (JST), Japan

ARTICLE INFO

Article history:
Available online 16 December 2010

Keywords:
R8
IRQ
Topology control
Clathrin-mediated endocytosis
Caveolae-mediated endocytosis
Macropinocytosis
Caveolae
Topology
Polyethylene glycol

ABSTRACT

It was recently reported that liposomes modified with octaarginine (R8) and its analogue peptide (IRQRRRR: IRQ) are taken up by 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, uptake was evaluated in the presence of some specific inhibitors of these processes. 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 by the ligand affects the contribution of the entry pathway, depending on the uptake mechanism.

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 the Tat peptide enhanced the cellular uptake of various macromolecules, the uptake mechanism remains to be elucidated [4–6]. It is likely that the cellular uptake pathway for 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 exclusively 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 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 a cellular uptake inducer for liposomes. Because of its stearyl moiety, it can easily be incorporated into the lipid bilayer, and results R8 being displayed on the surface of liposomes (R8-Lip). Furthermore, it has been demonstrated that the uptake of R8-Lip changes depending on the density of the peptide. Liposomes modified with a low density of STR-R8 (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 into the lipid bilayer. In contrast to R8-Lip, 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.

* Corresponding author. Address: Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan. Tel.: +81 11 706 3919; fax: +81 11 706 4879.
E-mail address: harasima@pharm.hokudai.ac.jp (H. Harashima).
[1] Present address: School of Pharmacy, Bandung Institute of Technology (ITB), Jl. Ganesha No. 10, Bandung 40132, West Java, Bandung, Indonesia.
[2] These authors equally contributed to this study.

1381-5148/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.reactfunctpolym.2010.11.013

Another possible factor that affects cellular uptake is the flexibility of the ligand. A previous study showed that mobility of maltose-conjugated α-cyclodextrins in polyrotaxane structures contributes to an improvement in affinity to concanalin A [13]. Other studies have shown 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 a PEG spacer [14]. However, the effect of the topology of the ligand on the internalization mechanism is remains unknown in the case of 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-PEG_{2000}-Lip and IRQ-PEG_{2000}-Lip, respectively).

2. Materials and methods

2.1. Materials

Stearylated-octaarginine (STR-R8) was synthesized as described previously [15]. The 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-PEG_{2000}-DSPE and IRQ-PEG_{2000}-DSPE were synthesized using a single-step reaction of Mal-PEG_{2000}-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-PEG_{2000}-Lip and IRQ-PEG_{2000}-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-PEG_{2000}-DSPE, or IRQ-PEG_{2000}-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 to the lipid composition. Meanwhile, R8-PEG_{2000}-Lip and IRQ-PEG_{2000}-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, followed by incubation for 1 h at 37 °C in the presence or absence of inhibitors. The cells were washed three 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 effect of a PEG spacer on the unique uptake pathway (macropinocytosis for R8-Lip and caveolar endocytosis for IRQ-Lip) was the main focus of this study. Thus, we fixed the density of ligands at 5%, in which both R8-Lip and IRQ-Lip were taken up via the pathway of interest. The cellular uptake of R8 and the IRQ-modified liposomes was evaluated in the presence of inhibitors.

Concerning the density of ligand, stearylated ligands (STR-R8 or STR-IRQ), PEG-linked lipid-ligands (R8-PEG_{2000}-DSPE and IRQ-PEG_{2000}-DSPE) were incorporated when the lipid film was formed. Thus, it is plausible that almost all of the ligands were stably incorporated in the lipid envelope, since these ligands were included in lipid film composition. The size and zeta-potential of liposomes were determined and the findings 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) since DSPE-PEG_{2000} possesses one negative charge derived from the phosphate group. However, the 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. The reason for why the mean sizes of IRQ-PEG_{2000}-Lip and R8-PEG_{2000}-Lip were larger than PEG_{2000}-Lip is unclear. IRQ-PEG_{2000}-Lip and R8-PEG_{2000}-Lip possesses negative charges on the proximal surface of the liposome, and positive charges on the ligand, and appears to result in the formation of nearly neutral particles apparently. It is likely that inter-particle electrostatic interactions occurred between the positively charges on the ligand and the negative charge on the liposome surface. However, it is noteworthy that a certain degree of dispersion in size (±40 nm) was observed in each measurement. Therefore, we concluded that a difference of 60 nm in mean size has a minimal effect on the overall trends in cellular uptake.

To identify the contribution of various endocytic pathways, the uptake of R8-Lip into NIH3T3 cells, IRQ-Lip, R8-PEG_{2000}-Lip and IRQ-PEG_{2000}-Lip was evaluated in the presence of inhibitors for macropinocytosis (amiloride) [17], clathrin-mediated endocytosis (sucrose) [18] and caveolar endocytosis (filipin III) [19]. Uptake was determined using CLSM and after removing the surface-bound liposomes with a heparin wash.

A previously reported quantitative analysis using a fluorescence-activated cell sorter (FACS) demonstrated that the cellular uptake of R8-Lip was strongly inhibited (approximately 80%) while inhibition with a hypertonic buffer and filipin III was less effective (approximately 40% and >10%, respectively) [11]. Consistent with these previous quantitative evaluations, the uptake of 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. Thus, the confocal images

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.