Effect of Polyethyleneglycol Spacer on the Binding Properties of Nuclear Localization Signal-Modified Liposomes to Isolated Nucleus

Dai KURIHARA, a Hidetaka AKITA*, a Asako KUDO, a Tomoya MASUDA, a Shiroh FUTAKI, b and Hideyoshi HARASHIMA a

*Faculty of Pharmaceutical Sciences, Hokkaido University; Sapporo, Hokkaido 060–0812, Japan; and b Institute for Chemical Research, Kyoto University; Uji, Kyoto 611–0011, Japan.

Received February 2, 2009; accepted May 9, 2009; published online May 11, 2009

The nuclear delivery process is a crucial barrier to successful gene delivery, especially in non-dividing cells. We previously proposed a novel strategy for the nuclear delivery of plasmid DNA (pDNA), in which the pDNA is encapsulated in lipid bilayers that had been modified with nucleus-targeting signals, including nuclear localizing signals derived from SV40 (NLS) or sugar units. In the present study, we report on an investigation of the effect of the topology of the liposome-modified NLS on its ability to bind to the isolated nucleus. NLS was directly attached to a liposome (NLS-Lip) by incorporating stearylated NLS (STR-NLS), or by modification with a polyethyleneglycol (PEG) spacer (NLS-PEG-Lip). NLS-unmodified liposomes (PEG-Lip) were used as a control. The liposomes, after labeling with 7-nitrobenz-2-oxa-1,3-diazole (NBD), were incubated with a cell homogenate derived from JAWS II cells, followed by isolation of the nuclear fraction by centrifugation. The PEG-Lip preparation showed negligible binding to the nucleus. In contrast, the binding of NLS-Lips to the nucleus gradually increased in a STR-NLS density-dependent manner. Interestingly, the binding of NLS-PEG-Lips to the nucleus is highly effective even at low density, suggesting that the presence of the PEG spacer is an important factor in improving the binding activity of NLS-modified liposomes to the nucleus. This information will be useful for the design of nucleus-targeting carriers.

Key words liposome; nucleus; binding; topology; polyethyleneglycol

The successful control of intracellular trafficking is prerequisite for the development of a gene delivery system. To date, efforts to overcome the rate-limiting processes have been extensive. To improve endosomal escape, pH-dependently membrane-fusogenic lipids 1) and/or peptides 2,3) or polycations with proton-sponge activity 4,5) have been integrated into carriers. In non-dividing cells, it is generally thought that the nuclear membrane is the ultimate barrier to be overcome, since it is composed of a double lipid bilayer structure, and mutual interactions between the cytoplasm and the intranuclear compartment are limited exclusively to transport through the nuclear pore complex (NPC). Small viruses (i.e. including Parvovirus 6) or Hepatitis B virus 7)), which are able to pass through the threshold size of the NPC (ca. 39 nm 8)) directly enter to the nucleus through the NPC, presumably aided by nuclear localization signals that are located on their capsid proteins. In contrast, adenovirus with a large genomic DNA (36 kDa) is approximately 90 nm in diameter, which exceeds the threshold size of the NPC. Nevertheless, it can achieve a high level of gene expression activity due to its highly efficient nuclear delivery. In the process of delivering adenovirus genome DNA to the nucleus, two rate-limiting steps are possible. First, adenoviral particles dock to the NPC. The subsequent binding of histone H1 to the adenovirus capsid, and import factors (i.e. importin 7) induce the disassembly of the adenovirus capsid structure, thus triggering the nuclear transfer of genomic DNA 9).

In a previous study, we reported on a nuclear gene delivery system that mimics the mechanism of adenoviral nuclear entry. In this carrier, pDNA was condensed with a polycation, followed by encapsulation with envelopes composed of lipid bilayers. For the targeting the NPC, the surface of the nuclear envelope was modified with nuclear localization signals derived from SV40 (NLS) or nuclear targeting sugars by incorporating stearylated NLS (STR-NLS) 10) or cholesterol-conjugated sugars 11) into the lipid film. As a result, gene expression activity was drastically enhanced, compared to that of the NLS- or sugar-unmodified carriers. To further enhance the nuclear targeting activity of the preparation and gene expression efficiency, the binding activity of the lipid envelope to the nucleus needs to be further optimized.

In the present study, we evaluated the nuclear binding activity of liposomes modified with a NLS with and without a PEG spacer, in order to investigate the effect of the topology of the NLS on the liposome surface on its ability to bind to the nucleus.

MATERIALS AND METHODS

Egg yolk phosphatidycholine (EPC), cholesterol (Cho), distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), N-[(3-maleimide-1-oxopropyl)aminopropyl]polyethyleneglycol-carbamyl]distearoylphosphatidyl-ethanolamine (DSPE-PEG-Mal) and 7-nitrobenz-2-oxa-1,3-diazole labeled DOPE (NBD-DOPE) were purchased from Avanti Polar lipids (Alabaster, AL, U.S.A.). Stearyl NLS (STR-NLS) was synthesized as described previously 10). JAWS II cells derived from murine dendritic cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Other chemicals used were commercially available and were reagent grade products.

Conjugation of NLS to the DSPE-PEG-Mal (DSPE-PEG-NLS) Ten micromolars of NLS (PKKKRKVED-PYC) in water was mixed with 10 mM of DSPE-PEG-Mal to permit the cystein residue to react with the maleimide moiety via a Michael addition reaction. Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF)-MS spectroscopy
data were obtained on a Bruker MALDI-TOF-MS Reflex II instrument to analyze masses of DSPE-PEG-Mal and DSPE-PEG-NLS using acetonitrile:water = 3:7 with 0.1% of trifluoroacetic as the matrix solution, supplied with 10 mg/ml of dihydroxybenzoic acid with a crumb of NaCl, and 10 mg/ml of sinapinic acid, respectively.

Preparation of Liposomes Lipid films composed of EPC : Chol = 7:3 (total lipid content: 0.55 μmol) with 1 mol% NBD-DOPC, and 1, 3 or 5 mol% of DSPE-PEG, STR-NLS and DEPE-PEG-NLS were prepared by evaporating the chloroform. They were then hydrated with 1 ml of 10 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) for 10 min at room temperature. The hydrated lipid film was then sonicated using a probe-type sonicator to give NLS-unmodified liposomes (PEG-Lips), liposomes directly modified with the NLS (NLS-Lips) and liposomes modified with the NLS with a PEG spacer (NLS-PEG-Lips). The diameter and zeta potential of the MENDs were determined by electrophoretic light-scattering spectrophotometry (Zetasizer; Malvern Instruments Ltd., Malvern, WR, U.K.).

Binding Assay of Liposomes to the Nuclear Fraction JAWS II cells were maintained with cultured in α-MEM (minimum essential medium eagle) (GIBCO) containing 20% FCS, 4 mM L-glutamine, 1 mM sodium pyruvate and 5 ng/ml of GM-CSF. The cells were trypsinized by treatment with 0.25% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) for cell passage. For the binding study, 2 x 10^7 cells were suspended in 2 ml of Binding Buffer (20 mM HEPES, 110 mM KOAc, 3 mM NaOAc, 2 mM MgOAc, 0.5 mM EGTA-Na, 2 mM dithiothreitol, and 250 mM sucrose, pH 7.3 adjusted with 6 N KOH), which is conventionally used for nuclear import studies when digitonin permeabilized cells are used. The cells were homogenized 100 times with a dounce type homogenizer. A minimal volume of sample (2 μl) was mixed with trypan blue to determine the number of nuclei present. The homogenate was then diluted with Binding Buffer to a concentration of 1 x 10^6 nuclei/ml. A 540 μl aliquot of the homogenate was mixed with 60 μl aliquot of liposomes at a concentration of 0.55 mM of the total EPC/Chol lipid, and the resulting suspension was incubated at 25°C for 30 min (Ftotal). A 500 μl aliquot of the suspension was centrifugated at 100 g for 2 min. Four hundred microliters of the supernatant was further centrifugated at 700 g for 10 min at 4°C. The nucleus-enriched pellet was suspended in 100 μl of Binding Buffer (Fnuc). One hundred microliters each of Ftotal and Fnuc were mixed with Binding Buffer containing with 1% of Triton, and the fluorescence was measured using an excitation wavelength of 470 nm and an emission wavelength of 530 nm. The total fluorescence in Fnuc and Ftotal were calculated, and the nucleus-binding activity was then calculated as follows:

\[
\text{binding activity} = \frac{F_{\text{nuc}}}{4F_{\text{total}}} \times 100(\%)\]

Statistical Analysis Statistical analyses were performed by one-way ANOVA, followed by Student–Newman–Keuls test.

RESULTS AND DISCUSSION

In the present study, NBD-labeled liposomes modified with a NLS with or without a PEG spacer were applied to a cell homogenate. For the labeling, lipid derivatives of NBD were incorporated when the lipid film was formed, thus ensuring that the liposomes were stably labeled with NBD. The nuclear binding efficiency was determined by isolation of the nuclei, followed by fluorescence measurements. Non-fusogenic liposomes composed of EPC/Chol were used in the study, to avoid non-specific binding via electrostatic interaction or membrane fusion.

For the modification of NLS with a PEG spacer, DSPE-PEG-NLS was prepared by incubating a 1:1 (mol/mol) mixture of DSPE-PEG-Mal and NLS. MALDI-TOF-MS spectroscopy showed that the DSPE-PEG-NLS gave a unimodal peak at a high molecular position (obsd. Mw = 4391.77, Mf = 4440.40, as compared to the DSPE-PEG-Mal (obsd. Mw = 3000.94, Mf/Mn = 1.02, calcd. Mf = 2941.64) (Fig. 1), indicating that the synthesis of DSPE-PEG-NLS was successful. Inspections of the peak area of DSPE-PEG-Mal before and after the synthesis (Figs. 1a, b, respectively) indicated that more than 80% of the DSPE-PEG-Mal was conjugated with NLS. For the direct modification of liposomes with NLS, STR-NLS was incorporated into

Fig. 1. MALDI-TOF-MS Spectroscopy of the Synthesized DSPE-PEG-NLS

MALDI-TOF MS spectroscopy was performed on a Bruker MALDI-TOF-MS Reflex II instrument to analyze a DSPE-PEG-Mal (a) and DSPE-PEG-NLS (b) with a solution of acetonitrile : water = 3:7 with 0.1% of trifluoroacetic as a matrix, supplied with 10 mg/ml of dihydroxybenzoic acid with a crumb of NaCl, and 10 mg/ml of sinapinic acid, respectively.
the liposomes. We used STR-NLS since, in a previous study, we demonstrated that modification of STR-NLS on the surface of liposomes enhanced the gene expression of encapsulated pDNA in primary dendritic cells.10) Although DSPE-PEGS may be more useful in comparing the effect of the PEG spacer, it is plausible that almost all of the STR-NLS and DSPE-PEG-NLS were stably incorporated in the lipid envelope since STR-NLS and DSPE-PEG-NLS were incorporated when lipid film was formed.

The size and zeta-potential of the prepared liposomes are summarized in Table 1. All of the liposomes were of comparable size (approx. 90—130 nm). The zeta potentials of PEG-Lips are nearly neutral, and modification of the STR-NLS or DSPE-PEG-NLS increased the zeta-potential in density-dependent manner (Table 1). This indicates that the cationic NLS peptide is actually displayed on the surface of the liposome.

In the present study, liposomes were applied to a cell homogenate, prepared with Binding Buffer, which constitutes optimum conditions for nuclear import study by digitonin permeabilized cells. To evaluate nuclear binding specificity, liposomes were added to a cell homogenate that also contained various organelles. Since mitochondria are damaged when incubated at 37 °C, this incubation was carried out at 25 °C. Therefore, it is plausible to conclude that the NLS peptides were recognized by importin α/importin β in the homogenate, which is the desired form for interaction with the FG-repeat domain in nuclear pore complex.14,15) Since the size of liposomes (>91 nm; Table 1) is larger than threshold size of the NPC (ca. 39 nm), the liposomes became bound to the nucleus, but their translocation through the NPC was negligible.

Figure 2 shows the effect of NLS density on the binding activity of NLS-PEG-Lips and NLS-Lips to the isolated nucleus. Overall, the binding activity is not correlated with the Z-potentials of the liposomes. As shown in Table 1, the Z-potential of NLS-Lips (3%) is comparable to that for NLS-Lips (5%), but the binding activity of the latter was higher than that of the former. Moreover, the Z-potentials of NLS-PEG-Lips were generally lower than those of NLS-Lips whereas the nuclear binding of NLS-PEG-Lips is higher than NLS-Lips, when 1% of NLS was modified, and vice versa when 5% NLS was modified. Collectively, these data indicate that nuclear binding cannot be explained by electrostatic interactions between the nucleus and liposomes. Furthermore, it is noteworthy that its binding activity is completely lost when the Binding Buffer is replaced with an isotonic buffer (IB: 250 mM sucrose, 2 mM Tris–HCl pH 7.4, 1 mM EDTA) during purification of the nuclear fraction. Considering the fact that the NPC is a giant complex comprised of 30—50 proteins,16,17) it is plausible that the structure and/or function of the nuclear pore complex structure is perturbed under non-optimized buffer conditions. These data suggest that NLS-dependent binding is due to a specific interaction between NLS-bound importin and the NPC.

NLS-PEG-Lips show a high binding activity at a low density of NLS, whereas it reached a plateau (approximately 25% of the applied dose) at 1 mol% modification (Fig. 2). In contrast, the nuclear binding of PEG-Lips was very low (<5% of the applied dose) at a low density of NLS (1%), and gradually increased in an NLS density-dependent manner.

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPC/Chol</td>
<td>116±4.3</td>
</tr>
<tr>
<td>EPC/Chol DSPE-PEG 1%</td>
<td>109±2.3</td>
</tr>
<tr>
<td>EPC/Chol DSPE-PEG 3%</td>
<td>100±2.2</td>
</tr>
<tr>
<td>EPC/Chol DSPE-PEG 5%</td>
<td>113±17</td>
</tr>
<tr>
<td>EPC/Chol STR-NLS 1%</td>
<td>105±16</td>
</tr>
<tr>
<td>EPC/Chol STR-NLS 3%</td>
<td>87±11</td>
</tr>
<tr>
<td>EPC/Chol STR-NLS 5%</td>
<td>91±29</td>
</tr>
<tr>
<td>EPC/Chol DSPE-PEG-NLS 1%</td>
<td>108±2.6</td>
</tr>
<tr>
<td>EPC/Chol DSPE-PEG-NLS 3%</td>
<td>132±31</td>
</tr>
<tr>
<td>EPC/Chol DSPE-PEG-NLS 5%</td>
<td>124±34</td>
</tr>
</tbody>
</table>

Consequently, the binding activity of NLS-Lips (approximately 30%) exceeds that of PEG-Lips when 5 mol% of NLS was modified (Fig. 2). These results suggest that NLS-PEG-Lips binds to the isolated nucleus with a higher affinity, but a lower capacity compared with NLS-Lips.

One possible explanation for the higher affinity binding of NLS-PEG-Lips is that NLSs on these liposomes may bind multivalently to NPCs on the nuclear surface, because of the extensive flexibility of the NLS. In contrast, the poor flexibility of NLS in NLS-Lips would prevent multivalent binding, and would result in a one-to-one binding of liposome and NPC. Alternatively, importin a can more efficiently recognize the NLS attached to a liposome with a PEG spacer.

The question as to why the NLS-Lips shows a higher capacity is unclear. If NLS-PEG-Lips bind to the nucleus via the multivalent binding of NLSs to the NPCs, the binding sites (NPCs) may be more completely occupied. In contrast, the nucleus can accept a larger amount of NLS-Lips since one NPC binds to one liposome because of the poor flexibility of NLSs. Therefore, the total capacity of nuclear binding of NLS-Lips may be larger than that for NLS-PEG-Lips.

The collective findings herein demonstrate that modifying NLS with a PEG spacer is advantageous for the binding of liposomes to the nucleus with a low density of NLS. Optimization of NLS density, PEG length and topology may sat-
isfy the efficient docking of particles to the NPC, and the subsequent disassembly of the lipid envelope, which is prerequisite for achieving the adenovirus-mimicked nuclear delivery of pDNA.

Acknowledgements  This work was supported in part by the MEXT Grant-in-Aid for Young Scientists (A) and by Grants-in-Aid for Scientific Research on Priority Areas from the Japan Society for the Promotion of Science. We also thank Dr. Milton Feather for his helpful advice in writing the manuscript.

REFERENCES