Identification and evaluation of the minimum unit of a KALA peptide required for gene delivery and immune activation

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ABSTRACT

The KALA peptide (WEAKLAKALAKALAKHLAKALAKALKA) is an amphiphilic peptide that forms an α-helical structure at physiological pH. We previously reported that, when a pDNA-encapsulating liposomal membrane is modified with the KALA peptide, transgene expression and immune activation are facilitated in bone marrow-derived dendritic cells (BMDCs). However, the minimum unit of the KALA peptide and the importance of its secondary structure for these activities are not completely known at this time. We herein report on the identification of the minimum unit of the KALA peptide (short-KALA) required for activity, as determined by the stepwise removal of “K-A-L-A” units. We evaluated the activities of 4 types of short-KALAs by modifying pDNA-encapsulating multi-functional envelop-type nano devices (MENDs). Among the peptides tested, a short-KALA3 (WEAKLAKALAKALA) was the shortest KALA peptide that could form an α-helical structure, as well as to elicit transgene expression and immune activation in BMDCs. Furthermore, the function of the short-KALA3 as an inducer of cellular uptake was retained, while uptake was completely lost in more shortened versions of KALA (short KALA4), in that transgene expression and immunological activation were both completely lost. These collective data show that the KALA peptide must form an α-helical structure to induce cellular uptake in BMDCs.

KEYWORDS
Non-viral gene delivery, Peptides, Circular dichroism, Liposomes, Vaccine delivery, Vaccine adjuvants

Gene vectors

**ABBREVIATIONS**

BMDCs: bone marrow-derived dendritic cells, ssPalm: ss-cleavable proton activated lipid-like material, MENDs: multifunctional envelop-type nano devices,
INTRODUCTION

An amphiphilic α-helical peptide, composed of hydrophobic and hydrophilic residues, is one of the key components of a drug delivery system, especially for the delivery of nucleic acids, since they contribute to the cytoplasmic delivery of macromolecules. To achieve cytoplasmic delivery, two processes should be taken into consideration, namely, cellular uptake and endosomal escape. Concerning the former point, previous studies reported that an α-helical peptide functions as a cell penetrating peptide\textsuperscript{1-4} For example, Mihara and co-workers reported on the identification of α-helical peptides that contribute to an enhanced cellular uptake based on libraries of α-helical peptides that they prepared and the applications of the peptide for the efficient uptake of gold nanoparticles\textsuperscript{1,2,5}. In the second point, the concept of α-helical peptide-mediated endosomal membrane-destabilization was originally inspired by the pH-triggered endosomal escape of the influenza virus with a hemagglutinin (HA) function: a part of the HA changes its conformation from a random coil to an α-helix structure in the acidic compartment, and then plays a key role in the destabilization of endosomes\textsuperscript{6,7}. Wagner and co-workers reported on the conjugation of synthesized peptides derived from its N-terminus on poly-L-lysine as a gene carrier, which resulted in the successful demonstration of enhanced gene expression\textsuperscript{8-10}. Similarly, an artificial amphiphilic peptide such as GALA (WEAALAEALAEHLAEALAEALEALAA)\textsuperscript{11,12}, which also undergo structural changes to an α-helix structure under acidic conditions, has been synthesized and used for a gene carrier.
The KALA peptide (WEAKLAKALAKALAKHLAKALAKALKA) was developed by substituting the glutamate residue in the GALA peptide with a cationic amino acid (lysine) to produce a molecule that forms an α-helical structure at physiological pH\textsuperscript{13}. This substitution also directly leads to the formation of complexes with functional nucleic acids (i.e. plasmid DNA (pDNA), small interfering RNA (siRNA)), resulting in an enhanced cellular uptake and function in \textit{in vitro} cultured cells\textsuperscript{13-16}. In addition, when nanoparticles are modified with the KALA peptide (i.e. liposomes, micelles, inorganic particles), this contributes to a more efficient cellular uptake and endosomal escape of the nanoparticles and/or their cargo \textit{in vitro} and \textit{in vivo}\textsuperscript{17-21}.

We previously reported on the preparation of a lipid derivative of the KALA peptide (stearylated KALA: STR-KALA), that can be used to modify the liposomal surface via the insertion of a stearyl moiety into the lipid bilayer. The functionalized particles, referred to as multifunctional envelope-type nano devices (MENDs) encapsulating a pDNA/protamine complex, efficiently elicited transgene expression in mouse bone marrow-derived dendritic cells (BMDCs)\textsuperscript{22}. Furthermore, a microarray analysis revealed that the KALA-modified MENDs (KALA-MENDs) induced immune activation in BMDCs, via the stimulation of intracellular DNA sensors (i.e. stimulator of interferon genes (STING) / Tank-binding kinesin-1 (TBK1) pathway, absent in melanoma 2 (AIM-2) inflammasomes) and elicited antigen specific anti-tumor effects\textsuperscript{22,23}. For the future clinical translation of this system as a DNA vaccine, identifying the functionally minimum length of KALA that has the same characteristics as the original KALA would be desirable in terms of reducing manufacturing costs and improving the stability
of the drug formulation. Moreover, the importance of an α-helical structure in the transfection and immune-stimulative activity in BMDC is not fully understood.

Here, we report on the identification of the minimum unit of the KALA peptide required to allow transgene expression and immune activation against BMDCs. Furthermore, the secondary structure of the systematically shortened KALA peptide was characterized by CD spectra to clarify the relationship between the functions of the KALA peptide and its secondary structure.
MATERIALS AND METHODS

Materials

1, 2-dioleoyl sn-glycero-3-phosphoethanolamine (DOPE) and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Phosphatidic acid (PA) was purchased from Sigma (St. Louis, MO, USA). 1-(monomethoxy polyethylene glycol2000)-2, 3-dimyristoylglycerol (DMG-PEG2000) was purchased from the NOF Corporation (Kanagawa, Japan). 3, 3'-dioctadecyloxacarbocyanine perchlorate (DiO) was purchased from Invitrogen (Carlsbad, CA, USA). Protamine sulfate salmon milt was purchased from Calbiochem (Darmstadt, Germany). The chemical synthesis of the ss-cleavable and pH-activated lipid like material with vitamin E as a hydrophobic scaffold (ssPalmE) was reported previously\textsuperscript{24}. Stearylated KALA and stearylated short-KALA1, 2, 3, 4 (shown in Table 1) were custom-synthesized by Kurabo (Osaka, Japan) as described previously\textsuperscript{13}. Mouse recombinant granulocyte-macrophage stimulating factor (GM-CSF) was purchased from R&D systems. All other chemicals used were commercially available and reagent grade products. A CpGfree pDNA encoding luciferase (pCpGfree-Luc (0)) was prepared as described previously\textsuperscript{22}. The pDNA sample was purified with Qiagen Endofree Giga Kit (Qiagen, Valencia, CA, USA).

Male ICR mice (4-6 weeks old) and female C57BL/6J mice (6-8 weeks old) were obtained from Japan SLC (Shizuoka, Japan). The protocol for use of the mice was approved by the Pharmaceutical Science Animal Committee of Hokkaido University.
Preparation of MENDs encapsulating pDNA

MENDs prepared with ssPalmE (MEND<sub>ssPalmE</sub>) were prepared by the ethanol dilution method as described previously<sup>25</sup> with minor modifications. In a typical run, pDNA and protamine solutions (0.15 mg/mL and 0.11 mg/mL, respectively) were prepared in 10 mM HEPES solution (pH 5.3) to enable the ssPalm to develop a positive charge during the process of lipid dilution for efficient packaging. pDNA/protamine core particles at a nitrogen/phosphate (N/P) ratio of 1.1 were prepared by dropping 100 µL of the protamine solution into a 100 µL pDNA solution with vortexing. The resulting pDNA/protamine core particle was encapsulated into the envelope. The lipid composition of the MENDs was ssPalmE/DOPE/Chol/DMG-PEG<sub>2000</sub> = 3/4/3/0.3 (molar ratio). The lipids (330 nmol of total lipid, Lipid/DNA charge ratio of 4.1) were dissolved in 200 µL of ethanol. Under vortex mixing at room temperature, the lipid solution (200 µL) was rapidly diluted with an equal volume of the pDNA/protamine core particle suspension (50 vol% ethanol). The solution was further diluted by adding 3.6 mL of 10 mM HEPES solution (pH 5.3) to 5 vol% ethanol. The diluted solution was concentrated by ultrafiltration using an Amicon Ultra 4 (Millipore Corp. Billerica, MA, USA) by centrifugation at 1000 g at room temperature for 5 minutes. The suspended particles remaining on the upper column were diluted with 4 mL of 100 mM HEPES buffer (pH 7.4), and again concentrated by several centrifugations at 1000 g at room temperature for 5 minutes. Finally, the particles were diluted with 10 mM HEPES buffer (pH 7.4) to obtain the desired concentration. For labeling the lipid envelope, a 0.25 mol% solution of DiO was added in the lipid composition. Finally, stearylated KALA or various
stearylated short-KALA preparations (final concentration, 1, 3, 5 mol% of total lipid) were added to the
MENDs solution under vortexing to modify the surface of liposomal membrane. The diameter and
ζ-potential of the liposomes were determined using an electrophoretic light-scattering
spectrophotometer (Zetasizer; Malvern Instruments Ltd., Malvern, WR, UK).

**Preparation of BMDCs of mice**

Mice BMDCs were prepared as described previously. Briefly, bone marrow cells were cultured
overnight in RPMI-1640 medium containing 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium
pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (FCS). Non-adherent
cells were harvested and cultured in the same medium supplemented with 10 ng/mL GM-CSF. On
days 2 and 4, non-adherent cells were removed, and the remaining adherent cells were cultured in
fresh medium containing 10 ng/mL GM-CSF. On day 6, the non-adherent cells were used in
experiment as immature BMDCs.

**Transfection studies for evaluating gene expression and the production of IL-6**

BMDCs (4.0 × 10⁵ cells) were incubated with the KALA-modified MENDs (equivalent to 0.4 μg
pDNA) in serum-free medium for 3 hours. Medium containing 10% FCS was then added, followed by a
further 21 hours of incubation. GM-CSF, at a concentration of 10 ng/mL, was added to the medium
during the incubation. In the case of gene expression experiments, the cells were washed with PBS,
and solubilized with reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity in the cell
lysate was then measured by means of a luminometer (Luminescence-PSN; ATTO, Tokyo, Japan).

The amount of protein in the cell lysate was determined using a Bicinchoninic acid (BCA) protein assay kit (PIERCE, Rockford, IL, USA). In the case of experiments related to IL-6 production, the concentration of IL-6 in the culture supernatant was measured by an enzyme-linked immunosorbent assay (ELISA) supplied by R&D systems according to the manufactures’ protocol.

Measurement of CD spectra of KALA peptides with the MENDs

CD spectra were obtained using a model J-820 CD spectrometer (JASCO Corporation, Tokyo, Japan). Spectra were recorded at a scan rate of 500 nm/min with a time constant of 1 second, and the signal was then averaged over 8 scans. Molar ellipticities (per amino acid residue) (deg cm$^2$ dmol$^{-1}$) were calculated by the following formula: $\theta = (\theta) / l \times c \times N$, where $\theta$ is the molar ellipticity per amino acid residue, $(\theta)$ is the measured ellipticity (deg), $l$ is the optical path length (cm), $c$ is the molar concentration of the peptide (mol/L), and $N$ is the number of amino acid residue of the peptide. The spectra were obtained in the condition where 0.1 mg/mL peptide and 0.5 mM MENDs in 10 mM HEPES (pH 7.4) or 20 mM MES (pH 5.5, 6.5) was.

Hemolysis assay

Hemolysis assays were performed as described previously with minor modifications$^{27}$. Briefly, male ICR mice (4-6 weeks) were obtained from Japan SLC. Fresh red blood cells (RBCs) were collected from the ICR mice, and suspended in PBS (pH 7.4) or 20 mM malic acid/PBS (pH 5.5 or 6.5). The
RBCs suspension was mixed with the MENDs, and then incubated at 37 degrees for 30 minutes. After the incubation, the absorbance at 545 nm of the supernatant was measured after centrifugation at 500 g, for 5 minutes. The RBCs incubated with 0.5 % (w/v) Triton-X100, and without MENDs were used as a positive- and negative control, respectively. The % lysis is represented as the % of the absorbance of the positive control.

**Flow cytometric analysis of BMDCs**

To evaluate the uptake of the MENDs, BMDCs were incubated with MENDs that had been modified with 0.25 mol% DiO in serum-free RPMI-1640 for 1 hour. GM-CSF was added to the medium at a concentration of 10 ng/mL during the incubation. After the incubation, the cells were harvested by pipetting, followed by washing 2 times with RPMI-1640 medium, and 2 times with PBS containing 0.5% bovine serum albumin and 0.1% NaN₃. After washing, the BMDCs were analyzed by Gallios (Beckman Coulter, Inc. Brea, CA, USA).
RESULTS

Gene expression and immune activation by MENDs modified with the short-KALA

To identify the minimum unit of the KALA peptide needed to achieve both gene expression activity and immune activation activity, we designed four types of short-KALA peptides by the stepwise removal of a “K-A-L-A” unit from the original KALA peptide (referred as total-KALA) and then synthesized stearylated derivatives of the peptides for use in modifying the surface of the MENDs with them (STR-short-KALA1, 2, 3, 4; Table 1). In the original KALA-MEND\textsuperscript{22}, the STR-KALA was modified on a membrane-fusogenic lipid composed of DOPE/phosphatidic acid (PA). However, the use of this negatively charged lipid envelope as a platform for KALA-modification was not adequate, since it was difficult to stabilize the MEND particle, especially when shortened KALA preparations were used for the modification. The most likely reason for this is that the modification of 10 mol% short-KALA3 and 4 onto the lipid envelope (DOPE/PA) resulted in the formation of slightly negative particles that tended to aggregate due to a lack of electrostatic repulsion (short-KALA3), or negatively charged particles (short-KALA4), while modification using the short-KALA1 and 2, as well as the original KALA resulted in the formation of a stabilized particle, since these modifications conferred a negative-to-positive charge ratio. Therefore, a comparison of the transfection activity and immune-stimulative activity among the apparently positively and negatively charged particles, or aggregated particles would be counterproductive in terms of comparing the function of KALA derivatives, since cationic charge is
generally a key characteristic for cellular binding, and the subsequent uptake of the particle\textsuperscript{28}. Therefore, we used neutrally charged-MENDs composed of an SS-cleavable Proton-Activated Lipid-like Material with vitamin E ($\alpha$-tocopheryl succinate) as a hydrophobic scaffold (ssPalmE) as a platform for the pDNA carrier (MEND\textsubscript{ssPalmE})\textsuperscript{24,25,29}. A pDNA free from the CpG domain from the bacterial backbone and ORF was used as a reporter pDNA since a previous study demonstrated that the complete removal of the CpG domain from the pDNA improved the gene expression activity of the KALA-MENDs in BMDCs without any attenuation of innate immune activation\textsuperscript{22}. Modification of the MEND\textsubscript{ssPalmE} with 3 mol% of the KALA peptides resulted in the formation of positively charged MENDs regardless of the type of KALA peptide used (The physicochemical properties of MENDs are shown in Table 2). The transfection activity and immune-stimulative activities of the MENDs with various KALA peptides were then compared in BMDCs (Figure 1). The sizes of the KALA-modified MENDs that were measured in GM-CSF containing serum-free media were comparable to the values found in HEPES buffer (Supplementary Table 1). Based on these findings, we conclude that the particles were stable, and did not form aggregates during the transfection studies. As was observed in the case of MENDs prepared with DOPE/PA\textsuperscript{22}, modification with the total-KALA enhanced both activities, even when it was modified on the MEND\textsubscript{ssPalmE}. Furthermore, modification of MEND\textsubscript{ssPalmE} with short-KALA1, 2 and 3 resulted in an enhanced gene expression and immune activation activity to a level comparable to that for the total-KALA. On the other hand, these stimulative activities became marginal when the
short-KALA4 was used. Based on these results, the short-KALA3 (WEAKLAKALAKALA) was defined as a minimum unit of the KALA peptide needed for the activities.

**Measurement of CD spectra of the short-KALA peptides**

To clarify the mechanism for why the short-KALA3 was the minimum unit required for gene expression and immune activation, we evaluated the secondary structure of each short-KALA peptide in the presence of the MEND\textsubscript{ssPalmE} by CD spectroscopy (Figure 2). Consistent with a previous report\textsuperscript{13}, the CD spectra of the total-KALA showed the characteristic features of an $\alpha$-helical structure with negative extrema at 208 and 222 nm and positive extrema between 190-200 nm\textsuperscript{30,31} regardless of the pH. Similar features were observed in the case of the CD spectra of the short-KALA1, 2 and 3, although the intensities of each peak became gradually smaller depending on the length of the peptide.

In contrast, CD spectra of the short-KALA4 at physiological pH were completely different from the other peptides, with negative extrema between 190-200 nm suggesting the formation of a random coil structure\textsuperscript{30,31}. Although a significant change in the spectrum was observed at an acidic pH, the negative extrema were shifted slightly from 208 nm, typical of a signal for an $\alpha$-helical structure. Collectively, these results suggest that the peptides that were longer than the short-KALA3 form $\alpha$-helical structures at acidic and physiological pH, while the short-KALA4 failed to form this structure, regardless of the pH of the solution, indicating that the short-KALA3 was the minimum KALA peptide required to form an $\alpha$-helical structure in the presence of the MEND\textsubscript{ssPalmE}.
Membrane fusogenic activity and cellular uptake of the short-KALA modified MENDs

To gain further insights into the importance of an α-helical structure on the control of intracellular trafficking, membrane destabilization activity was compared among various MEND_{ssPalmE} preparations modified with KALA derivatives using a hemolysis assay. RBCs were incubated with MEND_{ssPalmE} modified with various KALA peptides under physiological and acidic pH conditions and the leakage of hemoglobin into supernatant was then measured (Figure 3). At neutral pH or slightly acidic pH (pH = 6.5), hemolysis activity was below the detection limit regardless of the type of modification without KALA-modification. While modification with the total-KALA peptide enhanced hemolysis activity, the absolute value was still nearly background. In contrast, hemolysis activity was drastically increased at a more acidic pH (pH = 5.5). The induction of hemolysis activity was observed even in the KALA-unmodified MEND_{ssPalmE} due to the function of the ssPalmE. These data suggest that the pH-triggered protonation of the tertiary amine structure in the ssPalmE resulted in the development of a positive charge, which, in turn, served as a driving force for the particles to interact with the negatively charged biomembrane. The MEND_{ssPalmE} modified with total-KALA elicited the most potent membrane disruptive activity under acidic conditions, and the activity became weak when the KALA peptide was shortened (Figure 3). A significant increase in hemolysis activity was observed when the total-KALA, short-KALA1, and the short-KALA2 peptides were used to modify the MEND_{ssPalmE}. Modification with the short KALA4 failed to enhance hemolysis activity. The most surprising result was that the hemolysis activity of the short-KALA3 modified MEND_{ssPalmE} was comparable to that for the
modification with the short-KALA3 might not be sufficient to accelerate a membrane fusion process, even when the short-KALA3 possessed an α-helical structure (Figure 2). These collective data indicate that the function of the short-KALA3 as an enhancer of gene expression and immune stimulator cannot be explained based on endosomal escape. We then evaluated the uptake of the 0.25 mol% DiO-labeled MENDsPalmE modified with various KALA peptides in BMDCs using flow cytometry. As a result, the original KALA-modified MENDssPalmE represented the highest uptake, while the activity decreased, depending on the deletion of the amino acids (Figure 4a). The most significant finding was that the uptake of the MENDssPalmE modified with the short-KALA4 had no significant effect on the cellular uptake process: uptake was minimal, and was comparable to that for the KALA-unmodified MENDssPalmE. Furthermore, in the case of the short-KALA3, modification enhanced the uptake of the MENDssPalmE in a density-dependent manner (Figure 4b). In contrast, modification with the short-KALA4 did not enhance cellular uptake, even though the particle developed an extensive positive charge in a density-dependent manner (Table 3). These data suggest that the α-helical structure in KALA, rather than the positive charge of the particle plays a key role in the efficient uptake of MENDs followed by gene expression and immune activation.
DISCUSSION

In this study, we further succeeded in identifying the minimum unit of the KALA peptide; short-KALA3 (WEAKLAKALAKALA) needed to form an α-helical structure. When the surface of the MEND_{ssPalmE} was modified with it, transgene expression and immunological responses were stimulated (Figure 1). The most significant functional difference between the short-KALA3 and the short-KALA4 (further shortened KALA without activity) was found in the cellular uptake process (Figure 4). Generally, positively charged nanoparticles are taken up by a variety of cells via electrostatic interactions with negatively charged proteoglycans on the cell surfaces\textsuperscript{28,32}. Inconsistent with this observation, the cellular uptake of the MEND_{ssPalmE} modified with the short-KALA4 remained low (nearly detection limit) regardless of its density, even when the ζ-potential increased to a level comparable to that for the short-KALA3 modified one. (Table 3). This observation indicates that the α-helical structure in KALA-peptide is a key determinant in terms of inducing the uptake of the particle by cells. These results are consistent with a previous report, in which Yang et al. reported that a cationic and amphiphilic α-helical peptide interacted with heparan sulfate and was then taken up by cultured cells\textsuperscript{33}. In addition to this report, Tchoumi et al. reported that an α-helical structure was prerequisite for the uptake of a cationic peptide, pituitary adenylate-cyclase-activating polypeptide (PACAP: an endogenous peptide necrohormone) in a glycosaminoglycan (GAG)-dependent manner. The PACAP assumes an α-helical structure in the presence of heparin, one of the GAG molecules on the cell surface. The uptake of PACAP was attenuated by knocking out the xylosyltransferase in
CHO-K1 cells, in which GAG expression on the cell surface was lost. Furthermore, the introduction of the D-enantiomer into the PACAP, which disrupts the α-helical structure, resulted in a decreased peptide uptake in wild-type CHO-K1 cells\textsuperscript{34}.

For the KALA peptide to be functional in terms of application to DNA vaccines, the formation of an α-helical structure at physiological pH is necessary, since this structure is required for the particle to enter the BMDCs. Thus, the stability of the α-helical structure should be taken into the consideration when a functional peptide and/or proteins are used in dendritic cells. In fact, much effort has been devoted to the use of various types of modifications in attempts to stabilize the α-helical structure of a functional peptide and/or protein\textsuperscript{35,36}. For example, Walensky et al. reported that the introduction of a hydrocarbon staple, which crosslinks amino acid residues in the apoptotic BH3 peptide to stabilize its α-helical structure, augments the apoptotic activity of the peptide\textsuperscript{37}. An alternate strategy is to increase the hydrophobicity of the surrounding environment, since an amphiphilic peptide, including the KALA peptide, prefers to form an α-helical structure in a hydrophobic environment\textsuperscript{13,38,39}. The surface of the lipid nanoparticle is critical, since the lipid bilayer provides a hydrophobic environment. In fact, Wyman et al. reported that the KALA peptide per se can form an α-helical structure when the peptide is mixed with a liposome suspension\textsuperscript{13}. To attach the KALA peptide to the liposomal surface more firmly, we conjugated a stearyl moiety to the KALA, which would permit the unit to be more easily incorporated into the lipid bilayer.
The short-KALA3 elicited activities for BMDCs which were comparable to those for total-KALA (Figure 1). However, the membrane fusogenic activity and the uptake of the MENDs modified with the short-KALA3 were significantly lower in comparison with the total-KALA (Figure 3, 4). These data indicate that poor cellular uptake and endosomal escape of the short-KALA3 modified MENDssPalmE might be complemented by the decrease in electrostatic interactions of the cationic peptide (KALA) and the DNA cargo, and/or mRNA. Hama et al. previously reported that cationic materials drastically inhibited the transcription and translation processes for delivered pDNA and/or transcribed mRNA by electrostatic interactions. Thus, the employment of short-KALA3 with a lower cationic charge (Table 2) would permit electrostatic interactions with the pDNA cargo and/or mRNA to be avoided, and thereby improve the transcription/translation processes. The lower degree of electrostatic interactions in short-KALA3 modified MENDs might contribute to the higher cytokine production, since it is plausible that the free-form of pDNA can readily trigger the activation of cytoplasmic DNA sensors. To clarify the precise mechanism responsible for this, further quantitative analyses of the intracellular trafficking (i.e. the amount of pDNA in nuclei, quantification of the transcribed mRNA) will be necessary.

One of the mysterious points is that the hemolysis activity of the KALA-modified MENDssPalmE was quite poor at physiological pH (7.4), even though the series of KALA peptides form α-helical structures (Figure 2). One plausible reason for this is that the KALA peptide becomes bound to the liposomal membrane at physiological pH via electrostatic interactions between its cationic sites and/or...
hydrophobic interactions with the helical structure. In this case, the KALA peptide might not be able to interact with biomembranes, such as the plasma membrane and the endo/lysosome membrane. On the other hand, at an acidic pH, the tertiary amines in the ssPalmE would be protonated, resulting in the development of a positive charge. In this case, the mutual association of the KALA peptide with the lipid envelope might then be prohibited via electrostatic repulsion. In this case, the KALA peptide would be predicted to readily associate with a biomembrane and destabilize it.

In conclusion, we succeeded in identifying the minimum unit of KALA peptide: the short-KALA3 as a functional device for use as a DNA vaccine carrier: inducing gene transfection and generating immune responses. Modification of pDNA-encapsulating MEND_{ssPalmE} with this peptide facilitated the transgene expression and the immune activation of BMDCs to the same extent as the original KALA peptide. Furthermore, this peptide is also the minimum one needed to form an α-helical structure on the MENDs, which can induce the uptake of the MEND_{ssPalmE}. We therefore conclude that the short KALA3-modified MEND_{ssPalmE} is a promising carrier for use in a DNA vaccine.

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**FIGURE LEGENDS**

**Figure 1.** Gene expression and immune-stimulative activity of various KALA peptide-modified MENDs. BMDCs (4.0 \times 10^5 \text{ cells}) were transfected with the various KALA peptide-modified or non-modified MENDs (equivalent with 0.4 \mu g pDNA encoding luciferase). After 24 hours, (a) Luciferase activity and protein concentration of the cell lysate were measured and the relative luciferase activity calculated. (b) IL-6 concentration of the supernatant was measured by means of ELISA. Data are the mean \pm SD (n=3-6). Statistical analyses were performed by the one-way ANOVA, followed by Bonferroni test. *P < 0.05, **P < 0.01, versus Non-modified.

**Figure 2.** CD spectra of various KALA peptides modified with MENDs at various pH conditions. The spectra were obtained under the condition where 0.1 mg/mL peptide and 0.5 mM lipid concentration of MENDs in 10 mM HEPES (pH 7.4), 20 mM MES (pH 5.5, 6.5). Total-KALA: corner dotted line (-----), short-KALA1: round dotted line (--------), short-KALA2: short-dashed line (- - - -), short-KALA3: long dashed line (---), short-KALA4: solid line (-----).

**Figure 3.** Membrane fusogenic activity of various KALA peptides-modified MENDs. RBCs were incubated with the various KALA peptide-modified MENDs at various pH conditions for 30 minutes at 37 degrees. The values are represented as relative values of the positive control acquired by Triton X-100 treatment. Data are mean \pm SD (n=3). Statistical analyses were performed by one-way ANOVA,
followed by Bonferroni test. **P < 0.01 (at pH 7.4), †P < 0.05 (at pH 6.5), ††P < 0.01 (at pH 5.5) versus Non-modified at respective pH conditions.

**Figure 4.** Uptake of various KALA peptide-modified MENDs. BMDCs (4.0 × 10^5 cells) were transfected with (a) 3mol% total-KALA or short-KALA peptides-modified (b) 1, 3, 5 mol% short-KALA3 or short-KALA4 peptide-modified MENDs labeled by 0.25 mol% DiO (equivalent with 0.4 µg pDNA). After 1 hour, BMDCs were harvested and the fluorescence intensity measured (Geo Mean) by flowcytometer. Data are mean ± SD (n=3). Statistical analyses were performed by one-way ANOVA, followed by Bonferroni test. (a) **P < 0.01 versus Non-modified. (b) **P < 0.01 versus 1mol% modification.
Figure 1.

![Graph a](image1)

- Luciferase Activity (RLU/mg protein)
- IL-6 (ng/mL)

![Graph b](image2)

- total-KALA
- short-KALA1
- short-KALA2
- short-KALA3
- short-KALA4
- Non-modified
Figure 2.

![Graph showing molar ellipticity (deg cm² dmol⁻¹) at pH 5.5, pH 6.5, and pH 7.4 for different forms of KALA.](image)

- Total-KALA
- Short-KALA1
- Short-KALA2
- Short-KALA3
- Short-KALA4
Figure 3.
Figure 4.

![Bar chart showing cellular uptake (Geo Mean) for different modified and non-modified KALA proteins.](chart)

- **total-KALA**
- **short-KALA1**
- **short-KALA2**
- **short-KALA3**
- **short-KALA4**
- **Non-modified**

**Cellular uptake (Geo Mean)**

- **short-KALA3**
- **short-KALA4**

**1% 3% 5%**
Table 1.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Net charge</th>
<th>Number of residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>total-KALA</td>
<td>WEAKLAKALAKALAKHLAKALAKALKA</td>
<td>+ 6</td>
<td>27</td>
</tr>
<tr>
<td>short-KALA1</td>
<td>WEAKLAKALAKALAKHLAKALAKALA</td>
<td>+ 5</td>
<td>22</td>
</tr>
<tr>
<td>short-KALA2</td>
<td>WEAKLAKALAKALAKHLA</td>
<td>+ 4</td>
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</tr>
<tr>
<td>short-KALA3</td>
<td>WEAKLAKALAKALA</td>
<td>+ 3</td>
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</tr>
<tr>
<td>short-KALA4</td>
<td>WEAKLAKALA</td>
<td>+ 2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Size (d.nm)</td>
<td>ζ-potential (mV)</td>
<td>PdI</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>------------------</td>
<td>--------</td>
</tr>
<tr>
<td>total·KALA</td>
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<td>20±3</td>
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<td>short·KALA1</td>
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<tr>
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<tr>
<td>short·KALA3</td>
<td>137±2</td>
<td>8±3</td>
<td>0.09±0.01</td>
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<tr>
<td>short·KALA4</td>
<td>145±1</td>
<td>7±1</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>Non-modified</td>
<td>131±3</td>
<td>-5±1</td>
<td>0.07±0.01</td>
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Table 2.
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<th>peptide</th>
<th>Modification rate</th>
<th>Size (d.nm)</th>
<th>ζ-potential (mV)</th>
<th>PdI</th>
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<tbody>
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<td>short-KALA3</td>
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<td>−1±1</td>
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</tr>
<tr>
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<td>3mol%</td>
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<td>0±1</td>
<td>0.09±0.03</td>
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<tr>
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<td>3mol%</td>
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<td>7±1</td>
<td>0.10±0.01</td>
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