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Peptide vector for gene delivery with high affinity for phosphatidylserine

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Abstract: Since phosphatidylserine (PS) is known to translocate to the external face of the plasma membrane when the cell membrane becomes disordered, we decided to focus our attention on PS as a target molecule for gene delivery. In this paper, the novel peptide Td3701 was designed, synthesized, and characterized for its physico-chemico-biological properties. Td3701 simultaneously exhibited both characters as a DNA carrier and a sensor probe for active targeting, which seemed to be triggered by structural changes in the presence of PS. This is a very unique character among nonviral vectors, and it is believed that Td3701 could be used for selective gene delivery. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: α-helix peptide; gene delivery; phosphatidylserine; targeting; transfection

INTRODUCTION

In gene therapy, viral vectors have been the most commonly used vectors owing to their high transfection efficiency. However, viral vectors are not only difficult to handle but also present some safety concerns such as undesirable immune responses, infections, or malignant and permanent changes in the cell's genetic makeup [1–3]. In order to avoid these problems, a variety of nonviral vectors such as cationic liposomes and synthetic polymers have been developed [4–6]. However, a drawback with these alternatives is that they show a rather low transfection efficiency, and many attempts have been made to increase their efficacy through the development of active-targeting-ability vectors.

In order to obtain active targeting formulations, specific antigens or receptors on the cell surface are used as target molecules, and modifications of vectors with a monoclonal antibody or a specific ligand have been performed [7–10]. Here, we targeted phosphatidylserine (PS) on the cell surface, and created a nonviral vector that possessed transfection ability with high affinity for PS.

PS is a component of the phospholipid bilayer of the plasma cell membrane and is normally located in the inner leaflet of the lipid bilayer [11]. This asymmetry is thought to be maintained by aminophospholipid translocase (ATPase that catalyzes the inward movement of aminophospholipids such as PS and phosphatidylethanolamine (PE)) [12], and inhibition of this enzymatic activity is thought to disrupt this asymmetry, leading to the translocation of PS to the outer leaflet of the plasma membrane.

Translocation of PS to the outer leaflet of the plasma membrane is a marker of apoptosis [13,14]. It is also observed that activated platelets expose PS on the cell surface, and the resulting surface-exposed PS strongly propagates the coagulation process [15]. That is, blood coagulation factors possess either a calcium dependent or independent PS-binding region, and this region contributes to the efficient anchoring of coagulation proteins on the PS-exposed cell surface [16,17]. Among these proteins, factor VIII (FVIII) recognizes the PS-rich membrane via the carboxy-terminal C2 domain without the need for calcium [18–20], and a study using synthetic peptides revealed that a region within the amino acid sequences 2303–2332 was critical for PS recognition [21].

In addition to apoptosis and the coagulation process, PS exposure has been observed on tumor cells [22–25], and also under certain conditions during activation of inflammatory cells and injured cells [26,27]. These findings implied that PS could serve as a target molecule in disordered cells, and active targeting was possible by using vectors with high affinity for PS.

In the present study, we attempted to create a new peptide vector of DNA designed on the basis of the amino acid sequence of the carboxy-terminal C2 domain of FVIII, which simultaneously possessed transfection ability and an active targeting profile (Figure 1).

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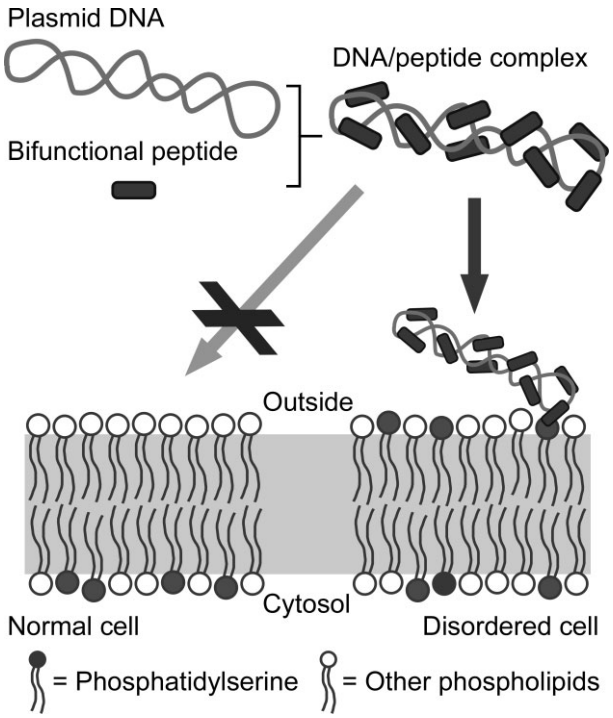

Figure 1 Scheme of gene delivery with active targeting profile for phosphatidylserine (PS). Translocation of PS takes place in membrane-disordered cells, and transport of DNA with affinity for PS occurs only in these PS-presented cells.

MATERIALS AND METHODS

Reagents

Recombinant human factor VIII (hFVIII) and monoclonal antibody ESH8 against hFVIII were purchased from American Diagnotica Inc. L-α-Phosphatidylserine from bovine brain (L-α-PS) and L-α-phosphatidylcholine from egg yolk (L-α-PC) were purchased from Sigma. HRP-labeled goat anti-mouse IgG was purchased from DAKO. Other reagents used for analysis were of reagent grade.

Preparation of peptides

Td3701 (T-R-Y-L-R-I-H-P-R-S-W-V-H-Q-I-A-L-R-L-R-Y-L-R-I-H-P-R-S-W-V-H-Q-I-A-L-R-S) and its derivative Td3701-scr (T-I-R-Y-R-P-S-H-Q-I-R-L- R-A-V-L-H-L-W-I-R-Y-R-P-S-H-Q-I-R-L-R-A-V-L-H-W-S) were synthesized on an automated peptide synthesizer (Model 433A, Applied Biosystems Inc.) and were purified by reverse phase high-performance liquid chromatography (RP-HPLC) on a C18 column (CAPCELL PAK C18AG120, Shiseido Fine Chemicals Co.), with a linear gradient established between 30 and 70% acetonitrile containing 0.01 N HCl for 15 min. The final products were identified by amino acid analysis and matrix-assisted laser desorption ionization mass spectrometry (Voyager DE-STR, PE Biosystems). The carboxy-terminal domain of hFVIII (2305–2332) (Y-L-R-I-H-P-Q-S-W-V-H-Q-I-A-L-R-M-E-V-L-G-C-E-A-Q-D-L-Y) was also synthesized, purified, and identified as described above.

Cell culture

Vero cells, a cell line of kidney epithelial cells from the African green monkey were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin. Cells were maintained at 37 °C with 5% CO_2 in a humidified incubator, and were passaged every 3–4 days. Cells were inoculated in 24-well plastic plates for the transfection assay at a density of 1.0×10^5 cells/well, and were cultured at 37 °C for 1 day.

Preparation of phospholipid vesicles

Phospholipids were dissolved in $CHCl_3$/methanol (2 : 1) and dried under a stream of N_2 gas. The dried lipids were hydrated in 20 mM Tris-HCl buffer, pH 7.4 using a bath-type sonicator. The suspension was sonicated for 20 min at 50 °C using a probe-type sonicator.

DNA binding assay

Tests were carried out by mixing 20 μg/ml plasmid DNA (pUC119; 3.16 kbp) and Td3701 under cation (peptide)/anion (DNA) ratios of 0, 1, 2, and 3, respectively, in 20 mM Tris-HCl buffer, pH 7.2 containing 150 mM NaCl. After a 30-min incubation at 37 °C, 5 μl of the solution was applied to 1% agarose gel containing EtBr, and was analyzed by electrophoresis.

Recognition of PS by synthetic peptides

Recognition of PS by synthetic peptides was evaluated using a competitive inhibition assay of hFVIII binding to PS, followed by a slightly modified method of Foster *et al.* [21]. Briefly, 100 μl of phospholipid cocktail composed of L-α-PS and L-α-PC in a 3 : 7 ratio dissolved in ethanol at a concentration of 10 μg/ml was placed into each well of a 96-well plastic plate (Immulon I, Dynatech Laboratories, Inc.). The plate was dried at 40 °C using a vacuum centrifuge evaporator (model EC-95C; Sakuma Seisakusyo, Ltd), and the wells were then blocked with 200 μl blocking buffer composed of 1% BSA, 154 mM NaCl, and 10 mM Tris-HCl buffer, pH 7.4, for 2.5 h at 37 °C. After the wells were washed with water, 100 μl recombinant hFVIII (1 μg/ml) in 154 mM NaCl, 5% BSA, and 10 mM Tris-HCl buffer, pH 7.4, in the presence or absence of Td3701, Td3701-scr, or hFVIII (2305–2332) was placed in each well, and incubated for 1 day at 4 °C. After washing three times with water and once with the washing buffer (154 mM NaCl, 10 mM Tris-HCl, pH 7.4), 100 μl monoclonal anti-hFVIII antibody (1 μg/ml) in 0.5% BSA, 154 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4 was added to each well and was incubated for 1.5 h at 37 °C. Using a general enzyme immunoassay (EIA) method, the amount of hFVIII bound to PS was evaluated using HRP-labeled goat anti-mouse IgG and 3,3′,5,5′-tetramethylbenzidine (TMB; 0.004% dissolved in 100 mM sodium acetate, 0.009% hydrogen peroxide, and 5% DMSO, pH 5.5) as the color developing reagent. Color development was quenched with 100 μl 1 N H_2SO_4, and absorbance at 450 nm was measured on a plate reader (THERMO max microplate reader, Molecular Devices).

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Preparation of DNA/Td3701 or DNA/liposome complexes

Reporter gene expression plasmid DNAs were constructed with firefly luciferase gene under the control of the SV40 early promoter. Plasmid DNA/Td3701, plasmid DNA/Td3701-scr, or plasmid DNA/cationic liposome complexes for transfection were prepared as follows: 16 μg luciferase expression plasmid DNAs was dissolved in 1 ml opti-MEM (Invitrogen), and 40 nmol/ml Td3701, Td3701-scr, or 256 μg/ml Lipofectin (Invitrogen) in opti-MEM. Equivalent volumes of plasmid DNAs and vector solutions were mixed, and were allowed to stand for 30 min at room temperature. This solution was then diluted with opti-MEM to obtain a concentration of 2 μg/ml plasmid DNA in the complex solution.

Evaluation of transfection efficiency

Vero cells were washed twice with saline, and then, 0.25 ml opti-MEM and 0.25 ml of the complex solution containing 2.0 μg/ml luciferase expression plasmid DNA in opti-MEM were added to each well. Cells were incubated for 5 h at 37 °C in 5% CO_2/95% air, washed once with growth medium, and cultured at 37 °C for 1 day.

Luciferase activity was measured using a luciferase assay kit (Promega). Following transfection, the cells were washed twice with saline. Subsequently, 200 μl Passive Lysis Buffer (Promega) was added to each well, and the mixtures were allowed to stand for 5 min at room temperature. Cells were then scraped from the culture plates, and the resulting cell homogenates were vortexed vigorously for 5 min in 1.5-ml plastic tubes. After centrifugation for 30 s at 10 000 g, luciferase activity in the supernatant was quantified using a luminometer (Arvo SX 1420 multilabel counter; Perkin Elmer). The amount of protein in the extracts was determined using protein assay reagents (Bio-Rad).

Measurement of circular dichroism spectra of the peptide

Peptide conformation was evaluated by circular dichroism (CD) spectra on a JASCO J-720W spectrometer using a quartz cell of 1.0-mm path length, at room temperature. Measurement of CD spectra of peptides at a concentration of 20 μM in the presence or absence of 5 mM SDS was carried out in 10 mM phosphate buffer, pH 7.0 containing 150 mM NaCl. Measurement of CD spectra of peptides at a concentration of 20 μM in the presence or absence of 1 mM phospholipid vesicles (PC alone or PC/PS = 7 : 3) was carried out in 20 mM Tris-HCl buffer, pH 7.4. The mean residue ellipticity is given in deg cm^2 $dmol^{-1}$.

RESULTS

Peptide design

Td3701 was designed on the basis of the amino acid sequence of human factor VIII (hFVIII), i.e. using the sequence of the region between aa. 2303–2320 located in the carboxy-terminal C2 domain (Figure 2). Peptide hFVIII (2303–2320) was combined in tandem, and

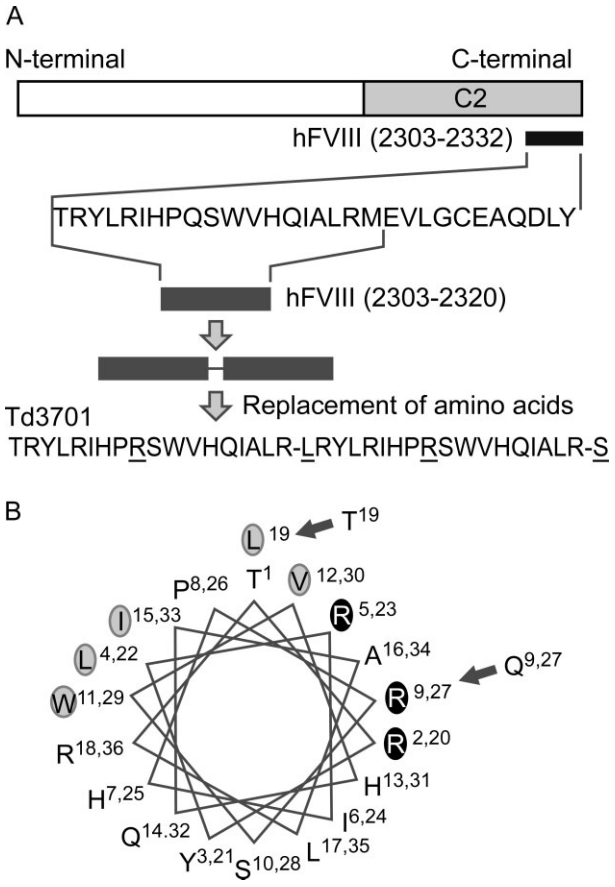

Figure 2 Amino acid sequence and predicted schematic structure of Td3701. (A): Design of Td3701 based on amino acid sequence of hFVIII; (B): Illustration of predicted α-helical wheels of Td3701. Replaced amino acids from the original sequence of hFVIII (2303–2320) are underlined.

Ser was attached to the carboxy terminal in order to increase peptide solubility. In the resultant peptide consisting of 37 amino acids, replacement of three amino acids (Q9->R, T19->L, Q27->R) was carried out to increase similarity with cationic amphiphilic α-helical oligopeptides [28]. Td3701-scr was designed to have the same amino acid composition but a different amino acid sequence of Td3701, and was expected not to fold into an α-helical structure.

Formation of the peptide/plasmid DNA complex

The ability of Td3701 to bind to plasmid DNA was evaluated according to general methods of electrophoretic mobility of peptide/DNA complexes on agarose gels. As shown in Figure 3, Td3701 suppressed the migration of plasmid DNA over a charge ratio of 2. This result indicated that Td3701 had the essential property of a cationic peptide, and had the ability to bind to negatively charged plasmid DNA. On the other hand, Td3701-scr also bound to, and suppressed the migration of, plasmid DNA (data not shown), since it had the same amount of positive charges as Td3701.

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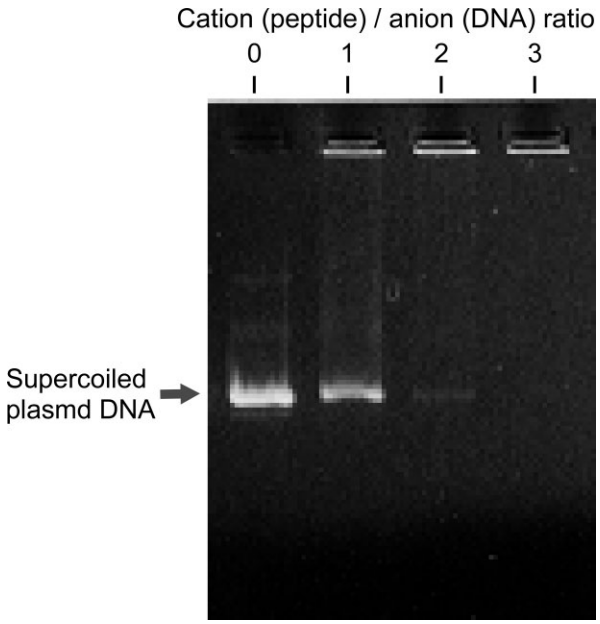

Figure 3 Agarose gel shift assay. Plasmid DNA was mixed with Td3701 at a cation (peptide)/anion (DNA) ratio of 0, 1, 2, and 3, and was analyzed by electrophoresis on 1% agarose gel.

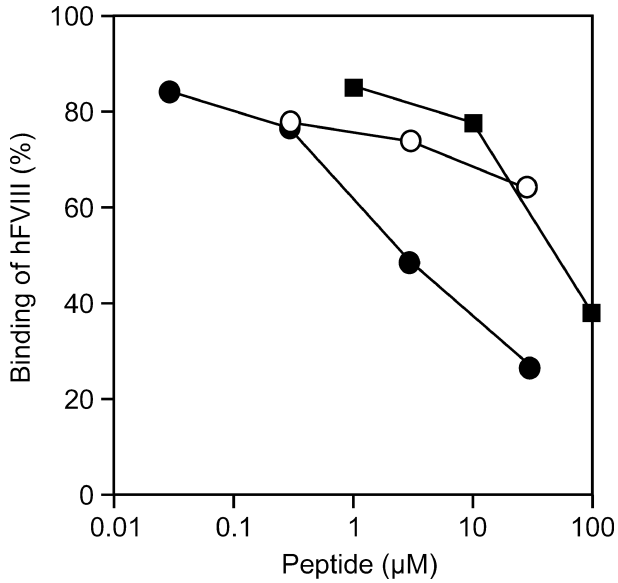

Figure 4 Recognition of PS by synthesized peptides. Evaluation of affinity for PS of the synthesized peptides was carried out by competitive EIA. Inhibition of the binding of hFVIII to PS was examined in the presence of Td3701 (closed circles), Td3701-scr (open circles), and hFVIII (2305–2332) (closed squares). The relative amount of hFVIII bound to PS was measured as described in 'Materials and Methods'.

Evaluation of affinity for phosphatidylserine

Recognition of PS by Td3701 was evaluated by competitive EIA. The amount of hFVIII bound to PS coated on the plastic plate was measured in the presence of Td3701 and Td3701-scr. hFVIII (2305–2332), which was previously reported as a PS-recognizing peptide, was used as the control peptide (Figure 4). Td3701 inhibited the binding of hFVIII to PS in the presence of BSA. Affinity of Td3701 to PS was stronger than that of hFVIII (2305–2332). On the other hand, Td3701-scr did not inhibit the binding of hFVIII.

Evaluation of transfection efficiency

Transfection efficiency of Td3701 was evaluated using luciferase expression plasmid DNAs in Vero cells in comparison with that of cationic liposomes (Lipofectin) (Figure 5). Td3701 showed a transfection efficiency similar to Lipofectin. On the other hand, Td3701-scr showed no transfection ability.

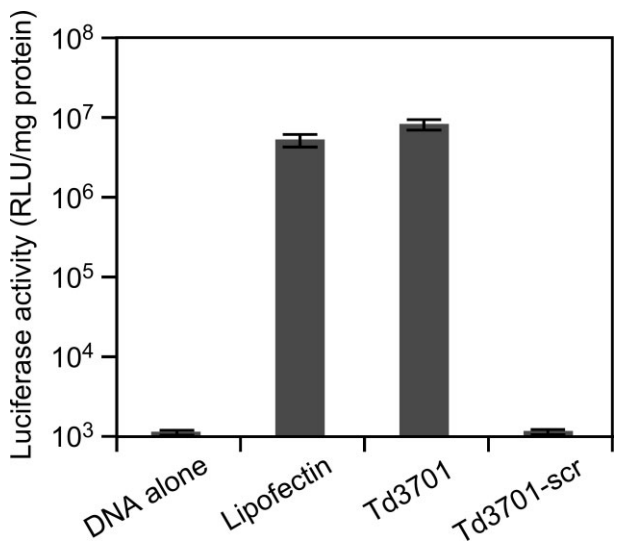

Figure 5 Evaluation of transfection efficiency. Transfection efficiency of Td3701 was examined and compared to those of Td3701-scr and the cationic liposome Lipofectin. Charge ratio of the peptide and plasmid DNA was 2.5. Concentrations of Lipofectin and plasmid DNA were 2 and 1 μg/ml, respectively. Luciferase expression plasmid was introduced into Vero cells, and transfection efficiency was evaluated by a luciferase assay.

CD spectra

Structural features of Td3701 were evaluated in the presence or absence of SDS (Figure 6(A)). Td3701 showed an α-helix CD pattern with double minima at 208 nm and 222 nm, only in the presence of SDS. However, Td3701-scr showed no α-helical structure, even in the presence of SDS (Figure 6(B)). Effects of phospholipids on structural changes of Td3701 and Td3701-scr were evaluated (Figure 7(A)). In the presence of PS-containing liposomes, a large valley at around 222 nm was observed, indicating that Td3701 adopted a partial α-helical structure. On the contrary, in the presence of PC-liposomes, Td3701 did not show an α-helical structure. On the other hand, Td3701-scr

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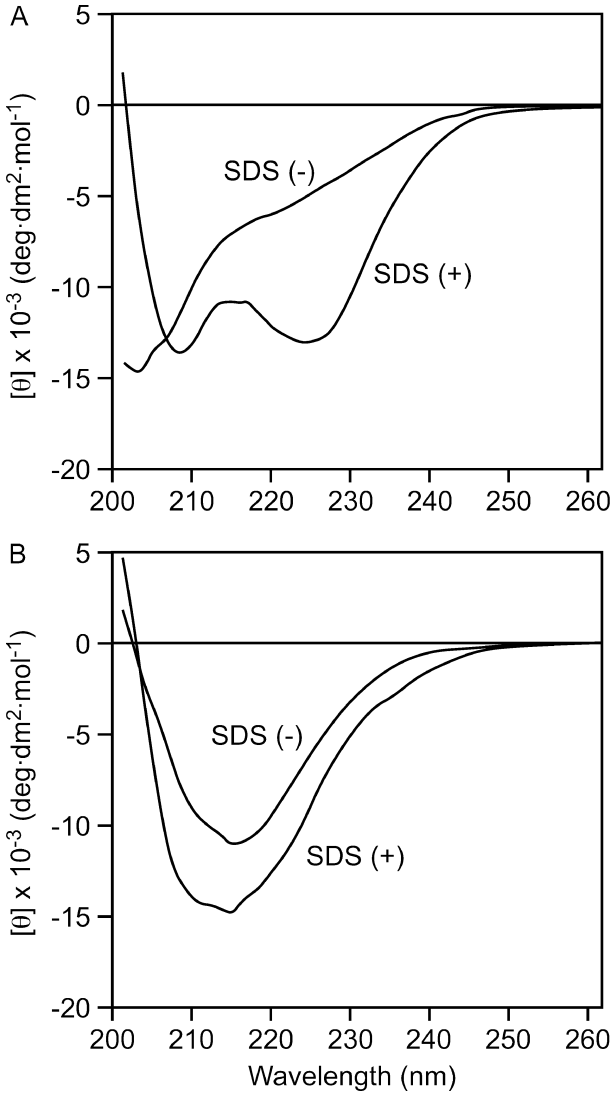

Figure 6 Structural changes of Td3701 and Td3701-scr in the presence of SDS. (A) CD spectra of Td3701 measured in the presence or absence of 5 mM SDS. (B) CD spectra of Td3701-scr measured in the presence or absence of 5 mM SDS.

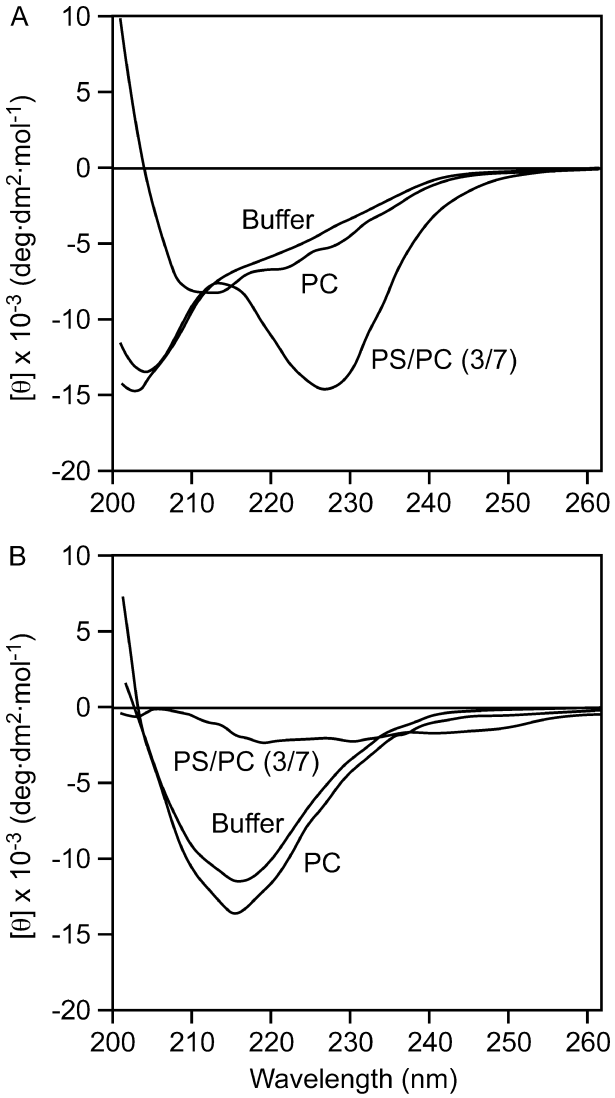

Figure 7 Structural changes of Td3701 and Td3701-scr in the presence of phospholipids. (A) CD spectra of Td3701 measured in the presence or absence of 1 mM liposomes composed of PC alone and PS/PC = 3/7. (B) CD spectra of Td3701-scr measured in the presence or absence of 1 mM liposomes composed of PC alone and PS/PC = 3/7.

showed no α-helical structure, even in the presence of PS-containing liposomes.

DISCUSSION

Dynamic movement of PS during translocation from the internal face to the external face of the lipid bilayer of the cell membrane when cell membranes are disordered suggests that disordered cells can be distinguished from normal cells by the presence of PS on the surface, and PS can be used as a marker for active targeting (Figure 1). In order to examine this possibility, nonviral vectors for gene delivery with an active targeting profile for PS have been developed. Here we describe Td3701, a 37-amino acid peptide vector with an affinity for PS.

At first, a peptide with the amino acid sequence of human factor VIII (hFVIII) containing residues 2303–2320 [hFVIII(2303–2320)] located in the carboxy-terminal C2 domain was selected as a PS-recognition peptide, according to previously reported studies [21]. This peptide sequence contained ten basic amino acids (6 Arg and 4 His), was shown to adopt an α-helical structure in the presence of detergent micelles [29], and appeared to have a basal character as a cationic amphiphilic α-helical oligopeptide, providing a promising candidate as a nonviral gene carrier because of its abilities for electrostatic binding to negatively charged DNA and for interactions with the phospholipid layer of the cell through the α-helical structure [30,31]. As a whole, hFVIII (2303–2320) was thought to offer

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the basic characteristics of a peptide with transfection ability and selective recognition of PS.

However, hFVIII (2303–2320) did not show any transfection ability (data not shown), so attempts to develop a new peptide (Td3701) were made on the basis of the amino acid sequence of hFVIII (2303–2320). That is, the amino acid sequence of hFVIII (2303–2320) was combined in tandem, and the hydrophilic amino acid Ser was attached to the carboxy terminal in order to increase peptide solubility. In the resulting peptide consisting of 37 amino acids, replacement of three amino acids (Q9->R, T19->L, Q27->R) was performed to increase electrostatic force, and to promote formation of cationic and hydrophobic faces of the predicted amphiphilic α-helical structure (Figure 2(A) and (B)).

Electrostatic interactions of Td3701 with plasmid DNA were examined, and as expected, migration of plasmid DNA was inhibited in response to an increase in the cation/anion ratio (Figure 3). In the calculation of the ratio, His was counted as a basic amino acid. However, under neutral conditions, His rarely possesses a positive charge, and therefore, the actual charge ratio may be close to 0, 0.6, 1.2 and 1.8 respectively, indicating that Td3701 and plasmid DNA bind almost quantitatively.

Td3701 is a vector with an affinity for PS that could recognize PS, and was evaluated by competitive EIA. The mixture of PS and phosphatidylcholine (PC) (PS/PC = 3 : 7) compared to PS alone was selected as the phospholipid solution because of the maximum efficacy of PS recognition by hFVIII (data not shown). As shown in Figure 4, Td3701 inhibited binding of hFVIII to the phospholipid layer with a higher affinity than that of hFVIII (2305–2332) which was previously reported as a PS-recognizing peptide [21]. Higher affinity might be induced by an increase in peptide length, and by an increase of the number of the reaction unit. On the contrary, Td3701-scr, which consisted of the same amino acids but with a different sequence to Td3701, did not show any inhibitory effect. Since the competitive assay was carried out in the presence of 5% BSA, nonspecific electrostatic interactions between positively charged peptides and negatively charged PS can be excluded. Therefore, considering that the net positive charge of Td3701-scr was the same as that of Td3701, the results clearly indicated that Td3701 recognized PS depending on its amino acid sequence. In fact, in the absence of BSA, not only Td3701 but also Td3701-scr showed inhibitory effects by nonspecific electrostatic interactions (data not shown).

Transfection efficiency of Td3701 was evaluated and compared to that of a cationic liposome (Lipofectin) using a firefly luciferase expression plasmid. As shown in Figure 5, Td3701 exhibited high transfection efficiency similar to that of Lipofectin. In accordance with results of the binding assay, Td3701 bound to DNA, and entered into the cells.

In order to understand mechanisms of PS recognition and transfection by Td3701, structural features of Td3701 were evaluated in the presence or absence of detergent/phospholipid micelles. The carboxy-terminal C2 domain of hFVIII (2303–2323) was shown to exhibit an α-helical structure in the presence of SDS [29], and in agreement with this, Td3701 exhibited an α-helical structure in the presence of SDS (Figure 6(A)). This result suggested that even hFVIII (2303–2320) combined in tandem, and the original potential of its α-helical structure was preserved in Td3701. Td3701 showed no structural change in the presence of PC-liposome and, in the presence of PS-containing liposomes, the α-helical structure formed (Figure 7(A)). These results proved that Td3701 showed an affinity to PS but not to PC, leading to a structural change from a random coil to an α-helix only at the face of PS. On the contrary, Td3701-scr did not adopt an α-helical structure even in the presence of SDS or PS-containing liposomes (Figures 6(B) and 7(B)). Rearrangement in the amino acid sequence reduced the α-helicity of the peptide. Although some induction of structural change of Td3701-scr was observed in the presence of PS as shown in Figure 7(B), this phenomenon could be explained by the absence of BSA in the reaction mixture, which might not have prevented nonspecific electrostatic interactions between positively charged peptides and negatively charged phospholipids.

CONCLUSIONS

The novel peptide Td3701, which was designed on the basis of the amino acid sequence of hFVIII, showed an affinity for PS and ability to deliver gene into cells exposing PS on the surface. CD measurements showed that Td3701 recognized PS with an α-helical structure, while Td3701-scr showed no α-helical structure, even in the presence of PS-containing liposomes. Td3701 is expected to be a multifunctional gene carrier for targeted gene delivery.

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