Improvement of peptide vectors for gene delivery with active targeting profiles for phosphatidylserine

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A cationic peptide, Td3701, which was derived from factor VIII that has affinity with phosphatidylserine (PS), showed efficient transfection ability for cells that express PS on the cell surface. PS is exposed on tumor cell surfaces therefore we have focused on PS as the target molecule for tumor specific gene delivery. In this article, to improve transfection efficiency and specificity in targeting tumor cells, some amino acid residues of Td3701 were replaced. The resulting peptide, Td3717, shows higher transfection efficiency (more than 30 times that of Td3701). The transfection efficiency was dependent on the amount of PS on the cell surface, suggesting that Td3717 bound with plasmid DNA could recognize PS on the cell surface. Td3717 is expected to be useful as an efficient gene carrier molecule specific to PS-presenting tumor cells. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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

Introduction

Targeted gene delivery and controlled release of the gene are critical properties of gene carrier molecules for successful gene therapy. To date, many chemically synthesized cationic compounds, such as cationic liposomes, polymers, and oligopeptides have been used as gene carrier molecules [1–5]. For targeted gene delivery, several peptide ligands such as the RGD peptide were coupled to the cationic compounds [6–8]. To enhance internalization of the DNA, cell-penetrating peptides such as the oligoarginine and TAT peptides were widely used [8–13]. For intracellular delivery of DNA, many peptides were used as fusogenic peptides to escape from endosomes and as signal peptides for delivery to the nucleus [8,13,14]. Furthermore, peptides can play an important role in controlled release of the DNA in the cells. McKenzie *et al*. [15] developed a cross-linked peptide containing multiple cysteine residues. The peptide formed complexes with DNA that were cross-linked via a disulfide bond. The cross-linkage of the peptides led to stabilization of the complex. After translocation into the cytosol, DNA release was triggered by disulfide bond reduction mediated by the cytosol's reducing environment [15–17]. Peptides were also used as a trigger to release DNA responding to intracellular signals. Oishi *et al*. [18] synthesized a peptide/polymer conjugate consisting of an uncharged main polymer chain and a cationic peptide, which included the substrate sequence of cyclic AMP-dependent protein kinase (PKA). The conjugate formed complexes with DNA. As activation of PKA in the cytosol phosphorylated the substrate peptide, an increase of anionic charge in the peptide weakened the interaction of the peptide with the DNA. The DNA was then released from the complex. Thus, peptides are powerful tools for the development of functional gene delivery systems.

Previously, we reported a peptide, Td3701, which acted as both a polycation for binding with DNA and a ligand for targeted delivery to disordered cells, e.g. tumor cells that express phosphatidylserine (PS) on the cell surface [19]. Td3701 was designed on the basis of the amino acid sequence of the carboxy-terminal C2 domain of coagulation factor VIII (FVIII), which is known to contribute to the efficient anchoring of FVIII on the surface of PS-exposed cells, such as activated platelets [20–23]. Td3701 showed affinity for PS, which is one of the components of the phospholipid bilayer of the plasma membrane of the cell. PS is normally located in the inner leaflet of the lipid bilayer, and translocation of PS to the outer leaflet of the plasma membrane would occur when this asymmetry maintained by enzymatic activity is disrupted. Such translocation of PS to the outer leaflet of the cell has been observed in tumor cells, and under certain conditions during activation of inflammatory

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and injured cells [24–29], Td3701 could be used as a ligand for selective gene delivery to disordered cells. However, the absolute gene transfection efficiency was lower than in conventional gene carrier molecules such as Lipofectamine2000™. In this study, we improved the transfection ability of Td3701 by optimizing the amino acid sequence of the peptide and studying the specificity to PS on the surface of the cells.

Materials and Methods

Reagents

Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylethanolamine (DOPE), L-α-phosphatidylinositol from bovine liver (L-α-PI), L-α-sphingomierine from egg yolk, and monoclonal anti-DNP IgE (SPE-1) were purchased from Sigma–Aldrich. DNP/BSA conjugate and FITC-labeled Annexin-V were purchased from Calbiochem and MBL, respectively. Lipofectin™, Lipofectamine2000™, opti-MEM, and NEAA (Nonessential Amino Acids Solution) were purchased from Invitrogen. Other reagents used for analysis were of reagent grade.

Synthesis and Purification of Peptides

Peptides Td3701 (TRYL-RIHP-RSWV-HQIA-LRLR-YLRI-HPRS-WVHQ-IALR-S) and Td3717 (TRYL-RLHP-RSWV-HQLA-LRLR-YLRL-HPRS-WVHQ-LALR-S) and its scrambled derivative, Td3717-scr (TLRY-RPSH-QLRL-RAVL-HLWL-RYRP-SHQL-RLRA-VLHW-S), were synthesized on an automated peptide synthesizer and purified by RP-HPLC on a C18 column (CAPCELL PAK C18AG120, Shiseido Fine Chemicals Co.) with a linear gradient established between 30% and 70% acetonitrile in 0.1% HCl for 30 min. The final products were identified by amino acid analysis and matrix-assisted laser desorption ionization mass spectrometry (VoyagerDE-STR, PE Biosystems).

Cell Culture

Vero cells (a cell line of kidney epithelial cells from African green monkey), F98 cells (rat glioma), Lewis lung carcinoma cells (mouse), and Meth-A cells (mouse fibrosarcoma) were cultured with Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serumFetal Bovine Serum (FBS), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin, respectively. The RBL-2H3 cells (rat basophilic leukemia) were cultured in minimum essential medium (MEM) containing 15% FBS, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin and were supplemented with NEAA (growth medium) at 37 °C. The cells were passaged every 3–4 days using a scraper. The SK-OV-3 (a cell line from human ovary cancer) cells were cultured with RPMI1640 medium supplemented with 15% FBS, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin. B16-BL6 cells (mouse melanoma) were cultured in MEM containing 10% FBS, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin supplemented with NEAA and vitamin solution at 37 °C. CASMC (coronary artery smooth muscle cell) was cultured with SmBM medium (basal smooth muscle cell media) containing 5% FBS enriched with human epidermal growth factor (hEGF), insulin, human fibroblast growth factor-β (hFGF-β), and GA-1000 (gentamicin/amphotericin) and NHDF (normal human dermal fibroblast) was cultured with FGM-2 (basal fibroblast growth medium) containing 2% FBS enriched with insulin, hFGF-β, and GA-1000. Cells were maintained at 37 °C with 5% CO_2 in a humidified incubator and passaged every 3–4 days.

Preparation of Phospholipid Vesicles

Phospholipids were dissolved in $CHCl_3$/methanol (2 : 1), and then dried under a stream of N_2 gas. The dried lipids were hydrated in 1 mM Phosphate Buffered Saline (PBS) (pH 7) using a bath-type sonicator. The suspension was further sonicated for 20 min at 50 °C using a probe-type sonicator.

DNA Binding Assay

The tests were carried out by mixing 20 µg/ml plasmid DNA (pBR322; 4.36 Kbp) and Td3717 at positive (peptide)/negative (DNA) ratios of 0, 1, 2 and 3 in 20 mM Tris/HCl buffer (pH 7.2) containing 150 mM NaCl. After 30 min at 37 °C, 5 µl of the mixtures was electrophoresed on 1% agarose gel containing EtBr, and the DNA bands were detected on UV lamp.

Complex Formation of Td3701, Td3717, and Liposomes with Plasmid DNA

Plasmid DNAs expressing the luciferase gene, pEF-Luc and pCMV-Luc, were constructed by inserting promoters (human elongation factor-1 promoter (hEF-1α) and cytomegalovirus (CMV) immediate-early promoter) into a luciferase expression vector. DNA complexes with the peptides and liposomes were prepared as follows; 16 µg of the plasmid DNA was dissolved in 1 ml opti-MEM (Invitrogen), and 40 nmol/ml of Td3701, Td3717, Td3717-scr, or 256 µg/ml of Lipofectin™ (Invitrogen) in opti-MEM was prepared. Equivalent volumes of the plasmid DNA were mixed with the peptide or Lipofectin™ solutions, and then 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 into Cells

Cells were inoculated in 24-well plastic plates at a density of 1.0×10^5 cells/well and cultured at 37 °C for 24 h. Cells were washed twice with saline, 0.25 ml of opti-MEM and 0.25 ml of the complex solution containing 2.0 µg/ml the plasmid DNA in opti-MEM were added to each well. The cells were then incubated for 5 h at 37 °C in 5% CO_2/95% air, and washed once with growth medium and cultured at 37 °C for 24 h. Luciferase expression was measured using a luciferase assay kit (Promega). After transfection, the cells were washed twice with saline. Subsequently, 200 µl of Passive Lysis Buffer (Promega) was added to each well and allowed to stand for 5 min at room temperature. The cells were then scraped from the culture plates and the resulting cell homogenates were vortexed vigorously for 5 min in a 1.5 ml plastic tube. After centrifugation for 30 s at $10\,000 \times g$, luciferase activities in the supernatants were quantified by a luminometer (Arvo™ SX 1420 multilabel counter, Perkin Elmer). The amounts of proteins in the supernatants were determined using a protein assay reagent (Bio-Rad).

Interactions Between Peptides and Phospholipids Membranes

Recognition of PS by the peptide was evaluated by surface plasmon resonance (SPR) using the BIAcore 3000™ system. Phospholipids were immobilized on the surface of an L1 chip using 0.2 mM

phospholipids solution followed by blocking solution (0.01% BSA/PBS). Peptide binding on the phospholipid membrane was measured by allowing 10 µM Td3717 or Td3717-scr solution in 0.01% BSA/PBS to flow over the chip, and dissociation was measured by initiating replacement with 0.01% BSA/PBS without peptide.

Measurement of Circular Dichroism Spectra of the Peptide

Peptide conformation was evaluated by 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 10 or 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 was given in deg cm^2 dmol^{-1}.

Activation of RBL-2H3 Cells

For evaluation of translocation of PS to the external face of the plasma membrane through stimulation, RBL-2H3 cells were plated in a 6-well plastic plate at a concentration of 1.5×10^6 cells/well. For evaluation of transfection efficiency after stimulation, RBL-2H3 cells were inoculated into 24-well plastic plates on at a concentration of 3.0×10^5 cells/well. Cells were, then, sensitized with anti-DNP IgE (100 ng/ml) at 37 °C in 5% CO$_2$/95% air [30]. After 16 h, the cells were washed twice with PBS, and degranulation buffer (0.1% BSA in phenol-red free RPMI 1640 (Invitrogen) supplemented with 2 g/l glucose) was added to each well. The cells were then stimulated with degranulation buffer containing 1 µg/ml DNP/BSA and incubated at 37 °C for 45 min in 5% CO$_2$/95% air.

Evaluation of Translocation of PS to External Surface of the Plasma Membrane

Cultured cells were detached using a cell scraper and suspended in 3 ml of PBS. The cell suspension containing about 8×10^5 cells was centrifuged at $200 \times g$ for 5 min and resuspended in 340 µl of binding buffer (MBL). Then, 10 µl of FITC-labeled Annexin-V was added to the cell suspension. The reaction mixture was incubated for 15 min in dark at room temperature. Fluorescence of bound FITC to the cell surface was measured by flow cytometry using CellQuest software (Beckton Dickinson).

Results and Discussion

Peptide Design

To improve the transfection efficiency of Td3701 [19], isoleucine residues in the peptide were replaced by leucine residues. Isoleucine residues disadvantage the formation of an α-helical structure of the peptide chain due to steric hindrance of the β-methyl group; contrastingly, the formation of an α-helical structure is favored by leucine residues. Since an amphiphilic α-helical structure is advantageous for DNA complex formation, endosome disruption, and overall gene transfection efficiency [31–34], we expected that replacement of isoleucine by leucine would improve the transfection efficiency. All four isoleucine residues in the peptide were replaced by leucine residues and the peptide was named Td3717 (Figure 1). As a control peptide, Td3717-scr (TLRY-RPSH-QLRL-RAVL-HLWL-RYRP-SHQL-RLRA-VLHW-S), which has the scrambled sequence of Td3717, was also designed and synthesized.

Formation of the Peptide/Plasmid DNA Complex

The binding ability of Td3717 to plasmid DNA was evaluated by assaying the electrophoretic mobility of the peptide/DNA complex on agarose gels [31]. As shown in Figure 2, Td3717 suppressed the migration of plasmid DNA over a positive (peptide)/negative (DNA) charge ratio of 2. Td3701 also showed suppression of DNA migration over the charge ratio of 2 [19], indicating that the replacement of isoleucine by leucine did not affect the DNA binding ability of the peptide.

Figure 1. Amino acid sequences (A) and predicted schematic α-helical structure (B) of Td3717 and Td3717-scr.

Figure 2. Agarose gel shift assay. Plasmid DNA was mixed with Td3717 at a positive (peptide)/negative (DNA) charge ratio of 0, 1, 2, and 3, and electrophoresed on 1% agarose gel containing EtBr. The DNA bands were detected using a UV lamp.

Evaluation of Transfection Efficiency

The transfection efficiency of Td3717 was compared with that of Td3701 and cationic liposomes, Lipofectin™ (DOTAP/DOPE = 1:1) and Lipofectamine2000™, using luciferase expression plasmid DNA and Vero cells. As shown in Figure 3, the transfection efficiency of Td3717 was 30 times higher than that of Td3701 and Lipofectin™. Compared with Lipofectamine2000™, the efficiency was six times lower. Td3717-scr did not show any transfection ability. Thus replacement of isoleucines by leucine residues was effective in improving the transfection efficiency. As the DNA binding ability of Td3717 was the same to that of Td3701, the internalization of the complex and/or the intracellular translocation process, such as endosome escape, must have been enhanced by the amino acid substitutions.

Evaluation of Affinity for Phospholipids Membranes

The interactions of Td3717 with several kinds of phospholipid membranes were evaluated by measuring SPR. The phospholipid membranes, consisting of PC alone and mixtures of 30% of PS, PI, PE, PG, or SM to 70% with PC (abbreviated to PC/PS, PC/PI, PC/PE, PC/PG, PC/SM, respectively), were prepared and immobilized on the sensor chip (L1). Solutions of Td3717 or Td3717-scr containing 0.01% BSA were introduced into the housing of the sensor chip. All phospholipids membranes showed increase in resonance signals when Td3717 flowed over the sensor chip, however, the signals decayed to the base line in the cases of PC alone, PC/PI, PC/PE, and PC/SM. Only in the cases of anionic membranes (PC/PG and PC/PS) was binding of Td3717 observed in the dissociation process (Figure 4(A)). This result suggested that Td3717 shows high affinity not only for PS but also for PG, meaning that Td3717 may show a
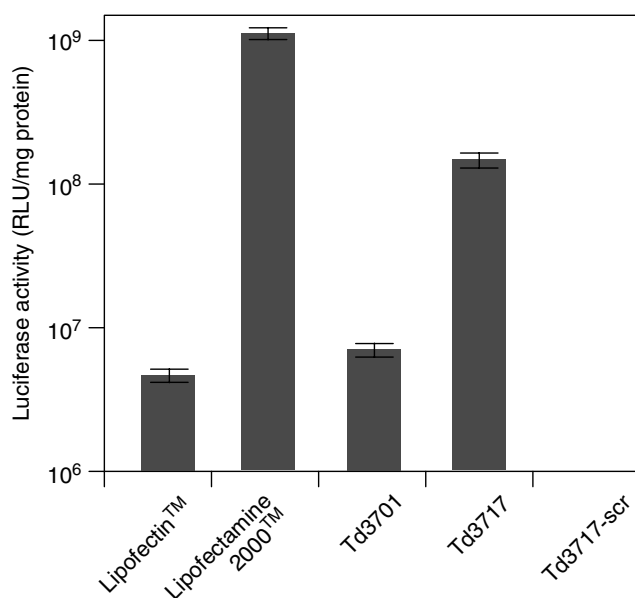
Figure 3. Transfection efficiency into Vero cells. Transfection efficiency of Td3717 was evaluated and compared with those of Lipofectin™, Lipofectamine2000™, Td3701, and Td3717-scr. The charge ratio of the carrier molecule and the plasmid DNA was 2.5. Concentrations of Lipofectin™/Lipofectamine2000™ and the DNA were 2 and 1 μg/ml, respectively. After 5 h incubation at 37 °C, the complex solution was replaced with fresh medium, and incubation was continued for 24 h at 37 °C. Luciferase activity in the cells was evaluated with a luciferase assay kit.
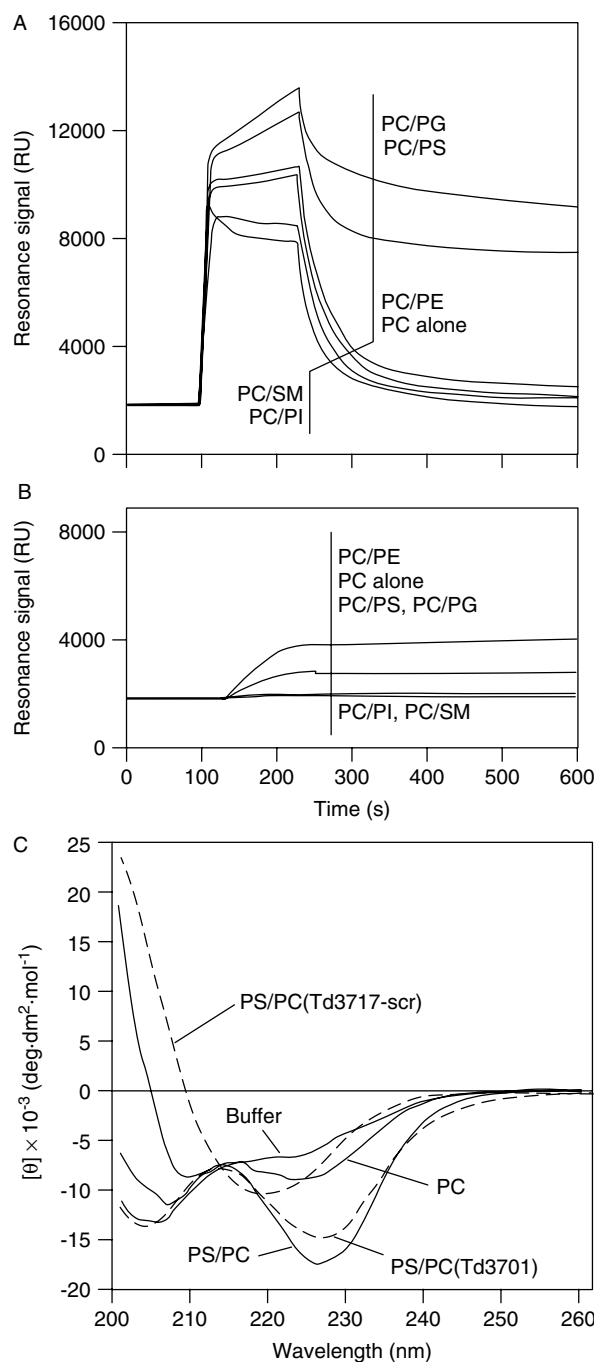
Figure 4. SPR sensorgrams showing adsorption and desorption of Td3717 (A) and Td3717-scr (B) on phospholipid membrane surfaces. Peptide binding on the phospholipid membrane was measured by allowing 10 μM Td3717 or Td3717-scr solution in 0.01% BSA/PBS to flow over the chip and dissociation was measured by initiating replacement with 0.01% BSA/PBS without peptide. Structural changes of Td3701 and Td3701-scr in the presence of phospholipids (C). CD spectra of Td3717 at a concentration of 20 μM were measured in the presence or absence of 1 mM liposomes composed of PC alone and PC/PS (*solid lines*). CD spectra of Td3717-scr (10 μM) and Td3701 (20 μM) were measured in the presence of 1 mM liposomes composed of PC alone and PC/PS (*dotted lines*).

selective affinity for anionic phospholipids. This characteristic does not compromise the strategy of the active targeting to disordered cells in the field of gene delivery using Td3717, because anionic phospholipids are rarely found in the phospholipid bilayer of the

plasma membrane of mammalian cell [34]. Therefore, among the anionic phospholipids, PS would still be a selective targeting molecule for Td3717-mediated gene delivery. In the case of Td3717-scr, weak bindings to PC/PE and PC membranes were observed, however, their strength was negligible compared with Td3717 (Figure 4(B)).

Structural features of the peptides were evaluated in the presence or absence of PS-containing liposomes (Figure 4(C)). In the presence of PS-containing liposomes (PC/PS = 7:3), Td3717 showed a large valley at around 222 nm, indicating that Td3717 adopted a partial α-helical structure. The valley of Td3717 was deeper than that of Td3701. The replacement of the isoleucine residues by the leucine residues actually strengthened the α-helical structure of the peptide, and affinity of the peptide with the PS-containing liposomes would be increased after the replacement. On the contrary, Td3717-scr showed β-sheet structure in the presence of the PS-containing liposomes. Furthermore, 10 μM of Td3717-scr was employed to measure the CD spectra while the other peptides (Td3717 and Td3701) could be measured at 20 μM because the use of 20 μM of Td3717-scr caused aggregation during the measurement, suggesting that

binding mode of Td3717-scr with the PS-containing liposomes was completely different from that of Td3717. In the presence of PC-liposomes, Td3717 showed weaker α-helical structure than the case of PS-containing liposomes, indicating that Td3717 had affinity with PS, and strong α-helical structure of the peptide was induced after the binding.

Selective Transfection Ability of Td3717 for PS Presenting Cells

In the measurement of SPR, Td3717 showed affinity for both anionic phospholipid membranes, PC/PG and PC/PS. To confirm the specificity of Td3717 to PS in the transfection system for cells, the relationship between the amount of PS on the cell surface and the transfection efficiency was examined.

RBL-2H3 cells were activated with immunogenic stimulation using anti-DNP IgE and DNP [30]. Annexin-V is well known as a detection molecule for apoptosis via its affinity for PS exposed on the cell surface. Therefore, the translocation of PS to the outside of the cells was easily detected and quantified with flow cytometry using FITC-labeled Annexin-V (Figure 5(A)). The transfection efficiency of Td3717 increased dramatically in the cells where translocation of PS had occurred (Figure 5(B)). In contrast, cationic lipids, Lipofectin™ and Lipofectamin2000™, showed no difference in the transfection efficiency before and after PS translocation.

The selective transfection ability of Td3717 was also examined using several kinds of cells that showed various amounts of PS on their cell surfaces. We employed Vero cells, SK-OV-3 cells, F98 cells, B16-BL6 cells, Lewis lung carcinoma cells, Meth-A cells, NHDF cells, and CASMC cells in addition to RBL-2H3 and activated RBL-2H3 cells. In Figure 6, the transfection efficiencies were plotted against relative PS amount on the cells surface (as evaluated by flow cytometry using FITC-labeled Annexin-V). A positive relationship between the expression of PS on the cell surface and the transfection efficiency was observed, suggesting that the transfection was mediated by affinity of Td3717 to PS on the cell surface.
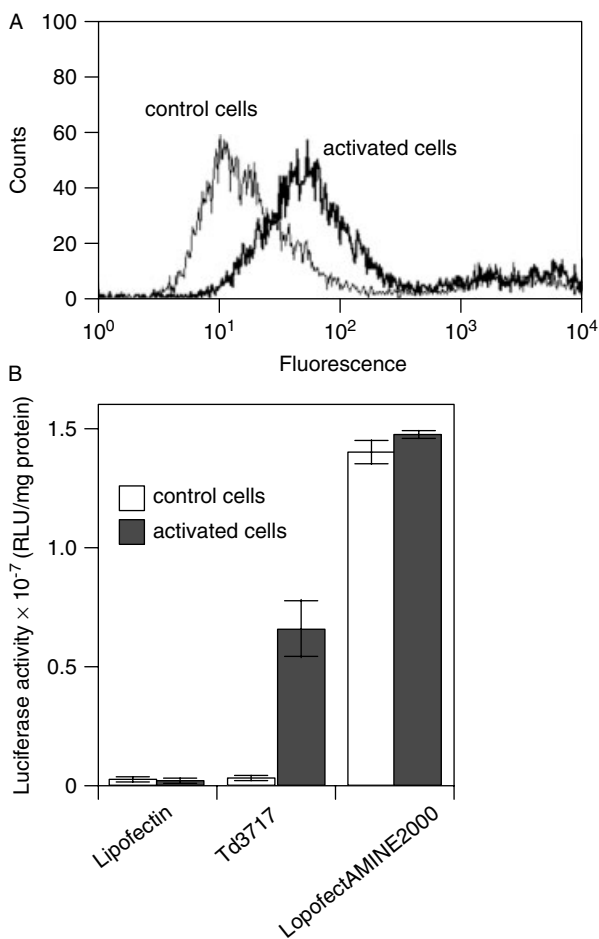
Figure 5. Translocation of PS on the surface of RBL-2H3 cells (A) and transfection efficiencies into normal RBL-2H3 cells and activated RBL-2H3 cells (B). RBL-2H3 cells were immunogenically stimulated using anti-DNP IgE for 16 h followed by addition of DNP/BSA for 45 min. Then, translocation of PS to cell surface was evaluated with flow cytometry using FITC-labeled Annexin-V. Transfection efficiencies to normal RBL-2H3 cells and activated RBL-2H3 cells were measured as described in Section "Materials and Methods".
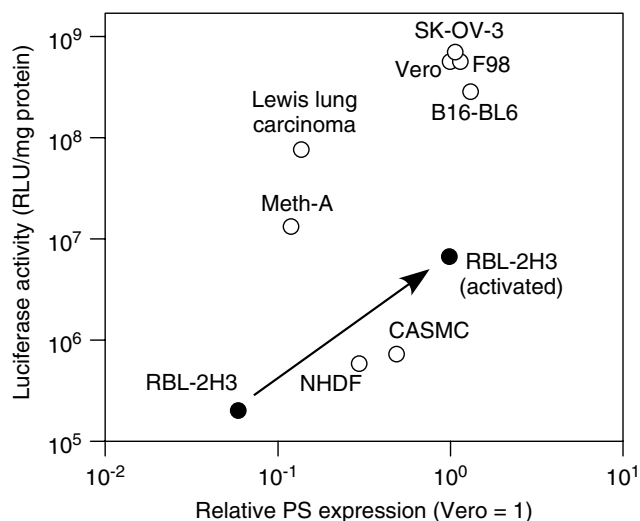
Figure 6. Relationship between PS expression on the external side of the plasma membrane and transfection efficiency. The amount of PS was expressed as a relative value against that of Vero cells.

Conclusions

PS is an attractive target molecule for active targeting, and Td3717, a peptide vector derived from Td3701, showed improved efficiency of transfection. In the test tube, Td3717 showed affinity for anionic phospholipids, i.e. both PC/PG and PC/PS. In an *in vitro* transfection system, a clear correlation between the amount of PS on the cell surface and the transfection efficiency was observed. Td3717 is a promising candidate for a nonviral vector for targeted gene delivery.

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