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Cellular signal-specific peptide substrate is essential for the gene delivery system responding to cellular signals

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ABSTRACT

Recently, there is a growing interest in the intracellular signal-targeting gene therapy or diagnosis, mainly by using the reaction of targeting enzymes with peptide substrates. In the present study, we proved the importance of target intracellular signal-specificity peptide substrate for intracellular signals-targeting gene therapy or diagnosis. Protein kinase C (PKC) was used as a trigger to activate the transgene expression. Two peptides, a positive peptide showing phosphorylation levels on several PKC isozymes (PKCα, βII, γ, ε, η, ζ, and ι/λ) and a negative peptide in which the phosphorylation site was destroyed by changing from serine to alanine, were designed. Moreover, two polymers possessing each peptide as a pendant chain, a PKC-responsive conjugate [PPC(S)] and a negative control conjugate [PPC(A)], were synthesized. After the introduction of complexes into cells or tissues, gene expression for PPC(S)/DNA complexes was higher that for PPC(A)/DNA complexes. However, no difference in gene expression between B16 melanoma tumors and normal skin tissues was identified. These results suggest that a peptide substrate specific to a target intracellular signal is very important for intracellular signals-targeting gene therapy or diagnosis.

Living cells contain numerous intracellular signal transduction pathways that respond to extracellular signals and regulate or modulate their gene expressions. In these intracellular signal transduction pathways, phosphorylation by protein kinases plays an important role in cellular growth and functions through activation of their target proteins. Some protein kinases are specifically and abnormally activated in the target diseased cells. Such hyperactivated protein kinases can be used for targeting diseased cells.[1]

Several studies have reported intracellular signal-targeting gene therapy or diagnosis, mainly by using the reaction of targeting enzymes with peptide substrates.[2] We recently proposed a novel strategy for disease cell-specific gene delivery system based on responses to intracellular signals such as protein kinase A,[3] caspase,[4] Ikappa-B kinase,[5] and protein kinase C (PKC).[6] These systems used peptide substrates specific to each intracellular signal.

PKC is a calcium- and phospholipid-dependent serine/threonine kinase. The PKC isozymes are classified into three subfamilies based on structural and activational characteristics: conventional or classic PKCs (cPKCs; α, βI, βII, and γ), novel or non-classic PKCs (nPKCs; δ, ε, η, and θ) and atypical PKCs (ζ, ι, and λ; PKCλ is the mouse homolog of PKCι). The activation of cPKCs requires diacylglycerol (DAG) as an activator and phosphatidylserine (PS) and Ca^{2+} as activation cofactors. The nPKCs are regulated by DAG and PS, but do not require Ca^{2+} for activation. In the case of atypical PKCs, their activity is stimulated only by PS, and not by DAG and Ca^{2+}. These PKC isozymes act directly and/or indirectly in signal transduction pathways of normal and transformed cells.[1] We recently succeeded in developing a PKCα-specific peptide (alphatomega). This peptide shows higher phosphorylation ratios for lysates from cancer cells and tissues, but much lower phosphorylation ratios for normal tissue lysates.[6a,7] Moreover, we developed the gene regulation system using this peptide and showed PKCα-responsive gene expression in cancer cell lines and tissues, but no expression in normal subcutaneous tissues.[6a]

In the present study, the PKC signal was used to prove whether a cellular signal-specific peptide substrate is essential for the intracellular signal-targeting gene therapy or diagnosis.

Two peptide substrates, LRVQNSLRRRR and LRVQNALRRRR, with a methacryloyl group at the amino-terminal were synthesized using an automatic peptide synthesizer according to standard

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Fmoc-chemistry procedures. After treatment with trifluoroacetic acid (TFA), the peptide was purified on an Inertsil ODS-3 column (250 × 20 mm, 3.5 μm; GL Sciences Inc., Tokyo, Japan) using a BioCAD Perfusion Chromatography system (Ikemoto Scientific Technology Co., Tokyo, Japan) and a linear A–B gradient at a flow-rate of 8 ml/min, where eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile.

For the phosphorylation of peptide substrates by PKC isozymes [all Sigma (Louis, MO, USA) except ι/λ (Upstate; Nihon Millipore, Tokyo, Japan)], the phosphorylation reaction was carried out in 50 μl of buffer (20 mM Tris–HCl at pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 100 μM ATP, 2.0 μg/ml DAG and 2.5 μg/ml PS) containing 30 μM peptide and 0.1 μg/ml of PKCα, βI, βII, and γ, but in buffer without $CaCl_2$ for PKCδ, ε, η, and θ, and without $CaCl_2$ and DAG for PKCζ and ι/λ. After incubation for 60 min at 37 °C, the reaction was stopped by addition of 50 μl of TFA and the samples were analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager DE RP Bio-Spectrometry Workstation (Applied Biosystems, Framingham, MA, USA) in the positive-ion reflectron mode. An α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml) was prepared in 50% water/acetonitrile and 0.1% TFA. The matrix and sample were mixed in a ratio of 1:1 (v/v), and a total volume of 1 μl of the mixture was applied to the sample plate. Following drying to allow crystallization, the mixture was analyzed by MALDI-TOF MS. The accelerating voltage used was 20 kV with a 100-ns extraction delay time. Typically, 100 laser shots were averaged to improve the signal-to-noise ratio. All spectra were analyzed using Data Explorer software (Applied Biosystems). The phosphorylation ratio was calculated as described previously.[8]

Acrylamide (13.2 mg, 0.19 mmol) and *N*-methacryloylpeptide (6 mg, 1.9 μmol) were dissolved in water, degassed with nitrogen for 5 min and then polymerized using ammonium peroxodisulfate (1.2 mg, 2.9 mmol) and *N,N,N′,N′*-tetramethylethylenediamine (1.63 μl, 5.8 mmol) as a redox couple at room temperature for 90 min. The water used in this study was distilled and purified using a Milli-Q water purification system (Millipore, Billerica, MA, USA). The synthesized sample was dialyzed against water overnight in a semipermeable membrane bag with a molecular weight cutoff of 50,000. The dialyzed sample was lyophilized and a final sample was obtained as a white powder, which was used as the polymer.

B16 melanoma cells were incubated in the absence or presence of the polymer (0–30 μg/ml) for 48 h in a 96-well plate. B16 melanoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) (all Gibco). The cells were kept in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. The conditioned medium in each well was replaced with 100 μl of flesh medium containing a cell proliferation reagent WST-1 {4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt} (Dojindo Laboratories, Kumamoto, Japan), and the cells were incubated for a further 3 h, before measurement of the absorbance at 450 nm. The percent cell viability was calculated by normalizing the absorbance of the treated cells to that of untreated cells.

Polymer/DNA complexes were prepared at cation/anion (C/A) ratios of 0.3 and 0.5. B16 melanoma cells were grown in 24-well plates for 24 h. After 24 h, the medium was changed to 500 μl of Opti-MEM (Gibco) and the complexes (50 μl) were added into wells. The wells were incubated at 37 °C for 6 h. After 6 h, the medium was changed to DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml), and was incubated for 24 h. Fluorescence micrograph of cells was obtained using a BZ-Analyzer (Keyence, Co., Ltd, Osaka, Japan).

Animal studies were performed in accordance with the Guidelines for Animal Experiments of the Kyushu University. Male 5-week-old BALB/c mice, weighting approximately 20 g, were used in this study. The dorsal side of each animal was shaved and inoculated subcutaneously with 1×10^7 B16 melanoma cells (Human Science, Osaka, Japan) in 100 μl of Hanks' balanced salt solution (Gibco). Tumors were allowed to grow to a mean diameter of approximately 8 mm.

Introduction of the polymer/DNA complex into B16 melanoma tumors or normal skin tissues was performed by direct injection. The polymer containing the positive or negative peptide substrate was mixed with a luciferase-encoding DNA (10 μg of pCMV plasmid DNA) at C/A ratios of 0.3, 0.5, and 1.0. Mice received a total of 100 μl of the polymer/DNA complex in 20 mM Tris–HCl buffer (pH 7.5) by direct injection into tumors or normal skin tissues. At 24 h, the tumors or normal skin tissues were assayed for luciferase activity. Following sacrifice of the mice, the tumors and skin tissues were excised and weighed. Next, the samples were homogenized in 1 ml of lysis buffer (100 mM Tris–HCl, pH 7.2, 0.05% Triton-X 100 and 2 mM EDTA), and the homogenate was centrifuged at 10,000*g* at 4 °C for 10 min. A 10-μl aliquot of the supernatant was used for measuring the chemiluminescence in a MiniLumat LB 9506 (EG & G Berthold, Wildbad, Germany) directly after adding 40 μl of the luciferin substrate. The results were presented as relative luminescence units (RLU)/mg total protein.

The total protein concentration of the lysate was assessed using the Bio-Rad Protein Assay Dye reagent (Bio-Rad Laboratories, CA, USA) with bovine serum albumin (BSA) as the standard. Briefly, working standard solutions containing 0.125–2 mg/ml BSA were incubated with the Bio-Rad Protein Assay Dye reagent. Following detection of their absorbance at 595 nm, calibration curves for the standard concentrations (0.125–2 mg/ml) versus the detector responses (absorbance at 595 nm) were obtained using a linear regression program. An aliquot of the extract was mixed with the Bio-Rad Protein assay Dye reagent and detected as described above. Its concentration was then calculated by reference to the calibration curve.

For lysate preparation from normal skin and B16 melanoma tumors, samples were excised from mice, weighed, and homogenized in 1 ml of buffer [20 mM Tris–HCl, pH 7.5, 250 mM sucrose and Complete™ protease inhibitor cocktail (EDTA-Free) (Roche, Tokyo, Japan)]. The homogenate was centrifuged at 1000*g* at 4 °C for 10 min and the supernatant was removed. After washing with 1 ml of buffer and recentrifuging, 1 ml of buffer was added into the precipitate. Samples were sonicated for 30 sec, then centrifuged at 5000*g* at 4 °C for 15 min, and the resulting supernatant was immunoblotted with anti-phosphoPKCα (Ser657) serum (Upstate) and the reacting proteins were visualized by a chemiluminescence.

Figure 1 shows a schematic illustration of the intracellular signal-responsive gene regulation used in this study. The artificial gene regulator possesses a neutral polymer backbone and cationic peptide side chains. This polycationic conjugate forms a tight complex with DNA through electrostatic interaction and suppresses its transcription efficiently due to the steric hindrance of the polymer backbone. When the complex is taken up to a target disease cell, in which the target protein kinase is extraordinarily activated or overexpressed, the peptide side chains in the complex are phosphorylated because the peptide is also designed as a specific substrate of the target protein kinase. This introduction of a phosphate anion cancels the cationic net charge of the conjugate, thereby weakening the electrostatic interaction between the conjugate and the DNA, and leading to the disintegration of the complex. This allows transcription factors access to the promoter region of the DNA strand, and gene expression is achieved.

Two peptide substrates, LRVQNSLRRRR and LRVQNALRRRR, were synthesized according to standard Fmoc-chemistry

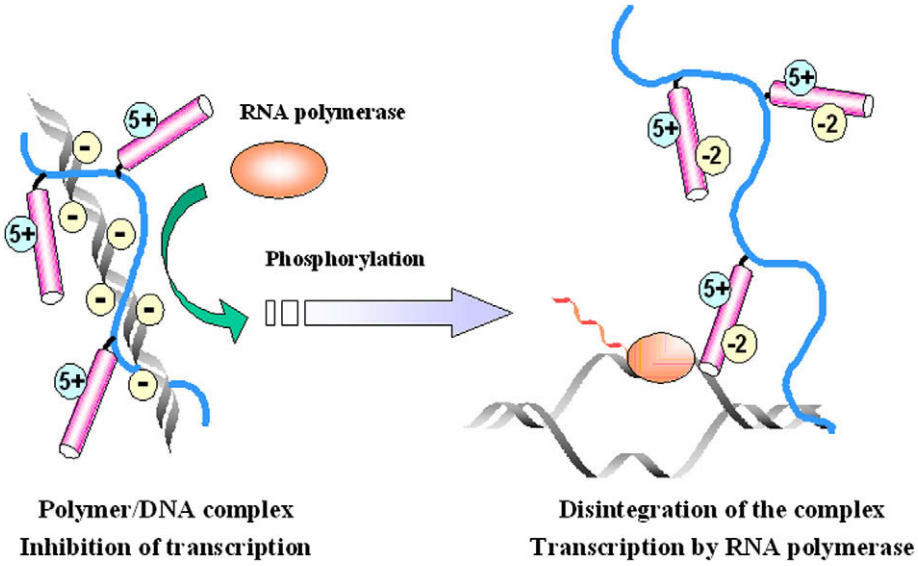

Figure 1. Scheme of gene delivery system responding to intracellular signals.

procedures. Phophorylation of peptide by PKC isozymes was identified using MALDI-TOF MS, since transfer of phosphate from ATP to the phosphorylation site serine can easily be evaluated due to an increase in the mass of the peptide by 80 Da.[8,9] Positive peptide (LRVQNSLRRRR) showed higher phosphorylation ratios (100%) for PKCα and ε than those for other PKC isozymes. Phosphorylation ratios for PKCγ and η were 32% and 34%, respectively (Fig. 2). On the other hand, no phosphorylated peaks were identified when the control peptides (LRVQNALRRRR) was used (data not shown).

On the basis of the phosphorylation data for the peptide substrate, a PKC-responsive polymer was designed. The polymer consisted of polyacrylamide as the main chain and the peptide substrate as side chains (Fig. 3). The content of peptide as the side chains of the polymer was estimated to be 2.0 mol % for the positive polymer [PPC(S)] containing the phosphorylation site serine and 1.5 mol % for the negative polymer [PPC(A)] that the serine in the peptide was substituted with alanine by using an elemental analysis, respectively. Since the peptide substrate has 5 cationic

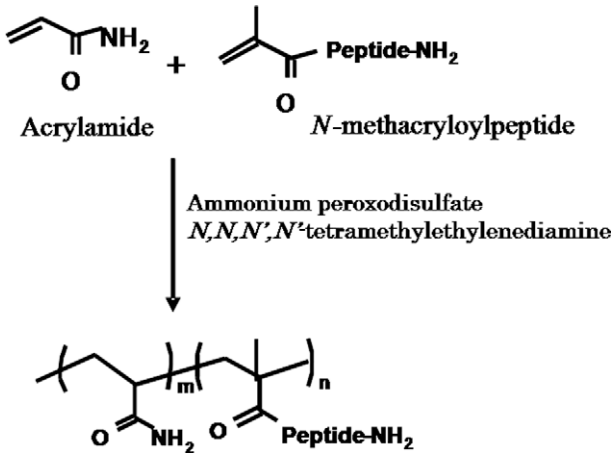

Figure 3. Synthetic scheme and chemical structure of polymer. The polymer was synthesized by polymerization of acrylamide and *N*-methacryloylpeptide using ammonium peroxodisulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

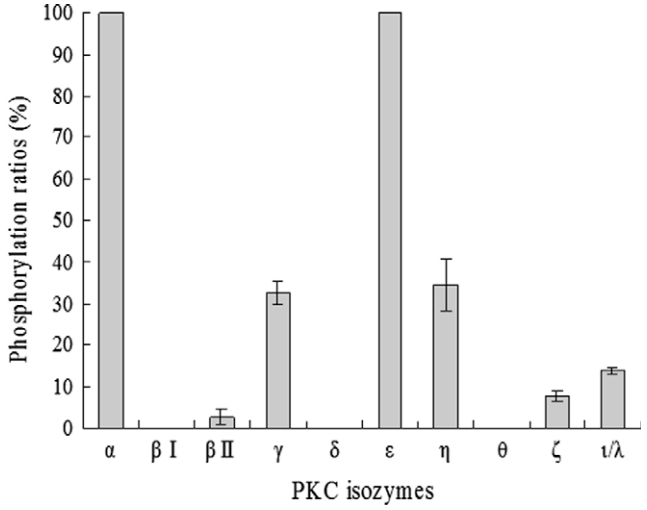

Figure 2. Phosphorylation ratios ($n = 3$) of peptide (LRVQNSLRRRR). The phosphorylation reaction was carried out in the presence of 0.1 μg/ml of PKC isozymes at 37 °C for 60 min and the phosphorylated products were identified by MALDI-TOF MS.

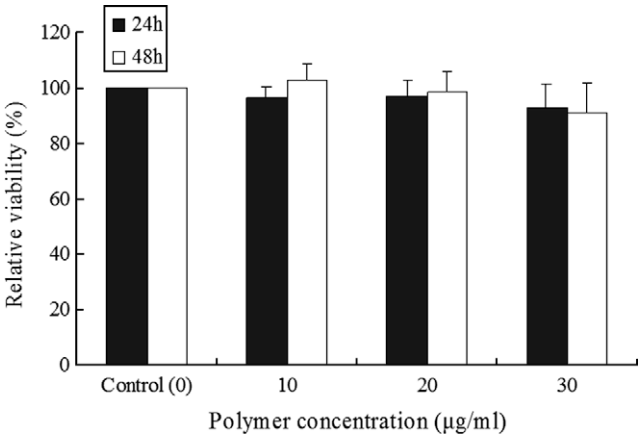

Figure 4. Cytotoxicity of the developed polymer toward B16 melanoma cells. Cells were incubated in the absence or presence of the polymer (0–30 μg/ml) for 48 h in a 96-well plate and the cell viability was measured using the WST-1 assay. The cell viability was calculated by normalizing the absorbance of the treated cells to that of untreated cells.

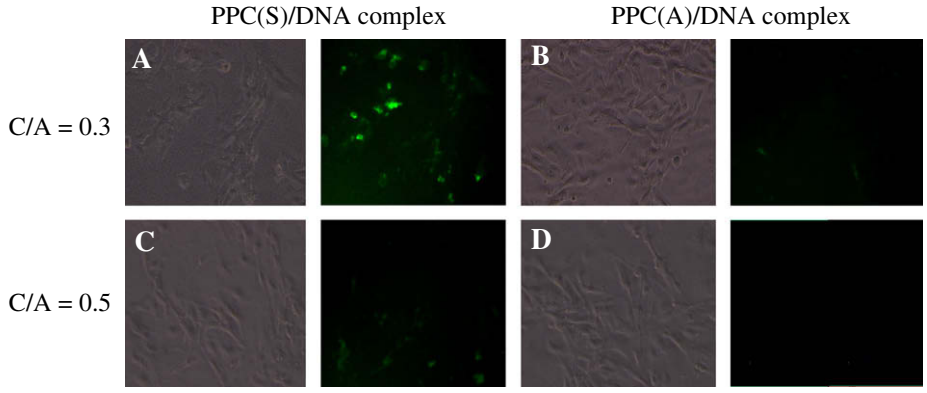

Figure 5. Polymer/DNA complexes prepared at C/A ratios of 0.3 [(A) and (B)] and 0.5 [(C) and (D)] were transfected into B16 melanoma cells in 24-well plates and were incubated at 37 °C for 6 h. After 6 h, the medium was changed to DMEM and fluorescence micrograph of cells was detected after incubation of 24 h. (A) and (C), PPC(S)/DNA complexes; (B) and (D), PPC(A)/DNA complexes.

amino acids (arginine), it was able to bind to anionic DNA sequences.

To identify the cytotoxicity of polymer toward cells, polymers at concentrations of 10–30 μg/ml were added into B16 melanoma cells. The assay results revealed that the developed polymer hardly affected B16 melanoma cell viabilities (>90%) in the concentration range of 10–30 μg/ml for 48 h. These results indicate no or very low cytotoxicity of the polymer toward cells (Fig. 4).

Polymer/DNA complexes at C/A ratios of 0.3 and 0.5 were transfected into B16 melanoma cells. Fluorescence derived from GFP was detected from cells transfected by PPC(S)/DNA complexes (Fig. 5A and C). Fluorescence levels were higher in a C/A ratio of 0.3 than in a C/A ratio of 0.5. In the case of transfection of PPC(A)/DNA complexes, however, very little fluorescence from a C/A ratio of 0.3 and no fluorescence from a C/A ratio of 0.5 were identified (Fig. 5B and D).

Moreover, polymer/luciferase-encoding DNA complex was delivered into tumors or normal skin tissues by direct injection. The luciferase activity of PPC(S)/DNA complex was over 10-fold higher than that of PPC(A)/DNA complex at C/A ratios of 0.3 and 0.5. Very low luciferase activities (<60,000 RLU/mg protein) were identified for a C/A ratio of 1.0. In the case of a C/A ratio of 1.0, the interaction between the polymer and the DNA was probably too tight to release the DNA (Fig. 6). On the other hand, similar luciferase expression was obtained from tumors and normal skin tissues.

Cationic polymers, such as polyamines and polyaminoacids, have been used as nonviral delivery systems for DNA. These cationic polymers must be able to form stable complexes with DNA.[10] Our polymer consists of a neutral main chain and peptide substrate side chains. The main chain has no charge, whereas each peptide substrate side chain contains cationic amino acids (arginine) and can bind to anionic DNA.

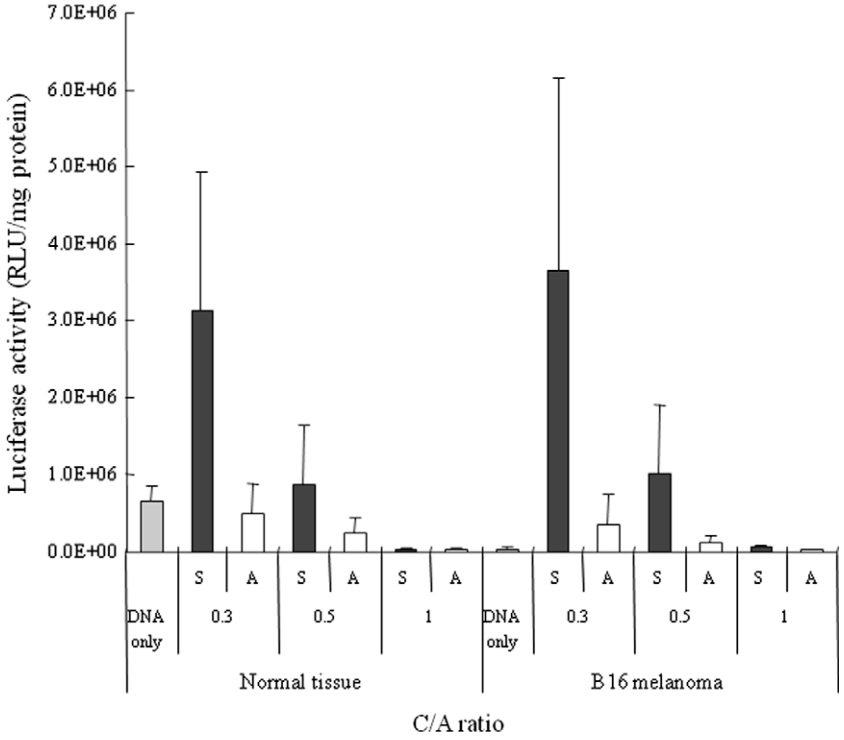

Figure 6. Luciferase assay of polymer/DNA complexes in tumors and normal skin tissues by direct injection ($n = 3$). At 24 h the samples were assayed for luciferase activity. The results are presented as the relative luminescence units (RLU)/mg total protein. S, the positive polymer [PPC(S)] containing the phosphorylation serine; A, the negative polymer [PPC(A)] that the phosphorylation site serine was substituted with alanine.

Recently, there has been an increasing interest in intracellular signals as therapeutic targets. PKC isozymes are important members of the major signaling pathways that regulate cancer cell differentiation, proliferation and survival. They have, therefore, attracted much attention as a potential target for anticancer therapies.[1d,11] In this study, we used PKC as a trigger to activate the transgene expression. A peptide (LRVQNSLRRR) used in this study showed phosphorylation levels on several PKC isozymes (PKCα, βII, γ, ε, η, ζ, and ι/λ).

When the complex at C/A ratios of 0.3 and 0.5 was introduced into B16 melanoma or normal skin tissues, the luciferase activity for PPC(S)/DNA complex was 10-fold higher than that for PPC(A)/DNA complex. However, similar luciferase expression was obtained from tumors and normal skin tissues. These results may be caused by peptide phosphorylation by other PKC isozymes. Skin tissue contains five PKC isozymes (PKCα, δ, ε, ζ, and η), while B16 melanoma has mainly PKCα.[12] The level of activated PKCα was higher in B16 melanoma than in normal skin tissue in the western blot test (Fig. 7). However, we found that the peptide can be phosphorylated by PKCα, ε, ζ, and η (Fig. 2). Thus, other PKC isozymes (PKCα, ε, ζ, and η) in normal skin tissues may have an effect on the phosphorylation of polymer/DNA complex, leading to luciferase expression. From these results, we suggest that a peptide substrate specific to a target intracellular signal is very important for the intracellular signals-targeted gene therapy or diagnosis. In spite of a growing interest in the intracellular signal-targeting gene therapy or diagnosis, however, there are few studies dealing with the importance of peptide substrate. Thus, our study may furnish with useful information for the nanoparticle design for the intracellular signal-targeting gene therapy or diagnosis.

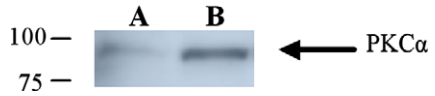

Figure 7. Western blot analysis of lysate prepared from (A) normal skin or (B) B16 melanoma tissues. The lysates were immunoblotted with anti-phosphoPKCα (Ser657) serum and the reacting proteins were visualized by chemiluminescence.

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