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# Design of a PKCδ-specific small peptide as a theragnostic agent for glioblastoma

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ABSTRACT

Glioblastoma is an aggressive malignant brain tumor that starts in the brain or spine and frequently recurs after anticancer treatment. The development of an accurate diagnostic system combined with effective cancer therapy is essential to improve prognosis of glioma patients. Peptides, produced from phage display, are attractive biomolecules for glioma treatment because of their biostability, nontoxicity, and small size. In this study, we employed phage display methodology to screen for peptides that specifically recognize the target PKCδ as a novel biomarker for glioma. The phage library screening yielded four different peptides displayed on phages with a 20- to 200-pM $K_d$ value for the recombinant PKCδ catalytic domain. Among these four phage peptides, we selected one to synthesize and tagged it with fluorescein isothiocyanate (FITC) based on the sequence of the PKCδ-binding phage clone. The synthetic peptide showed a relative binding affinity for antibody and localization in the U373 glioma cell. The kinase activity of PKCδ was inhibited by FITC-labeled peptide with an $IC_{50}$ of 1.4 μM in vitro. Consequently, the peptide found in this study might be a promising therapeutic agent against malignant brain tumor.



Brain tumors arise from abnormal cells in brain tissue. Tumor growth in the brain results in functional disturbances in the body and also induces dysfunction in neural circuitry. Glioblastoma is the most common type of brain tumor in adults, and ionizing radiation therapy is a well-known treatment method for glioblastoma [1]. However, radiation therapy frequently induces recurrence, which progresses as focal masses with potent tumorigenic activity [2]. Several studies have proven that conventional therapy has limited effect against tumor cells, and the major reason for reoccurrence is the existence of cancer stem cells (CSCs) [3,4].

CSCs possess self-renewal capabilities similar to those of stem cells [5]. The small population of CSCs in a tumor can differentiate, form a bulk population, and self-renew to maintain tumors [6]. Recent research suggests that CSCs in a tumor not only induce increased metastasis but also lead to drug resistance [7]. In glioblastoma, $CD133^+$ cells were first identified as cancer-initiating cells with stem cell properties that are necessary for the proliferation and self-renewal of the brain tumor [8,9]. Furthermore, there is evidence that the membrane protein CD133 is a marker for a subset of cells in the central nervous cell as well as glioblastoma and is a key factor in the progression and maintenance of glioma stem cells [10,11].

The protein kinase C (PKC) family consists of at least 11 members and plays an important role in the cell cycle [12]. In all cases, the PKC family consists of an N-terminal regulatory domain that contains a pseudo-substrate region and a C-terminal catalytic domain connected by a short hinge region [13]. The kinase activity of the PKC family resides in the catalytic region, which contains a conserved ATP- and magnesium-binding site that serves as a substrate-binding site for the phosphoacceptor sequence on the target protein.

The PKC family plays a key role in many human diseases, including diabetes [14], cancer [15], heart failure [16], and Parkinson's disease [17]. Among the PKC family members, PKCδ, a novel

*Abbreviations:* CSC, cancer stem cell; PKC, protein kinase C; TIC, tumor-initiating cell; cd-PKCδ, PKCδ catalytic domain; IPTG, isopropyl-1-thiogalactopyranoside; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; ELISA, enzyme-linked immunosorbent assay; TBST, Tris-buffered saline with Tween 20; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; FBS, fetal bovine serum; $IC_{50}$, 50% inhibition concentration; PKC-bp, PKCδ-binding peptide.

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class of PKC isoforms, was overexpressed in carcinogenic cells and mediated cancer cell migration, metastasis, and malignancy [18,19]. Emerging evidence points to an important role of PKCδ in the malignant phenotype of glioma. Activation of PKCδ is required for fractionated radiation-induced events, increases in the glioma stem cell population, and reductions in sensitivity to chemotherapeutic agents [20,21]. In addition, the population of CD133-positive tumor-initiating cells (TICs), which possess the regulatory properties of the glioblastoma population, was sustained by the regulatory mechanism of PKCδ [22]. Therefore, PKCδ is a potential biomarker to disturb the signaling of expression of CD133 on glioma cells and may have a therapeutic effect on glioblastoma stem cells by decreasing the metastasis and malignancy properties of target glioma cells.

To design a novel peptide-based diagnostic probe with inhibitory properties, we screened peptides via M13 phage display. Phage display is a standard technique for screening small peptides toward a specific target [23]. The display peptides on the phage can be selectively amplified and proliferated from a randomized phage library. This screening technique has facilitated the identification of peptide motifs that possess strong binding affinity to various targets [24]. As an alternative tool for a diagnostic system, the small peptides have several advantages over antibodies. Due to their smaller molecular weight, peptides possess a higher cell and tissue penetrating efficiency than antibodies [25]. Moreover, peptides have comparable binding affinity and specificity to antibodies. With these novel characteristics, peptides can be rationally designed and have various clinical applications [26,27].

In this study, we designed a small peptide-based theragnostic probe to target the PKCδ catalytic domain (cd-PKCδ) of glioma stem cells. The identified 12-amino-acid peptide showed strong binding affinity and specificity toward the PKCδ catalytic domain. Furthermore, the peptides possessed the ability to target and inhibit PKCδ. This study provides a novel peptide sequence for a theragnostic strategy to target glioma stem cells.

## Materials and methods

### *Materials*

mRNA was extracted using TRIzol reagent (Life Technologies, 15596-026). We used a polyclonal antibody against PKCδ purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and an Alexa Fluor 488 anti-rabbit secondary antibody (Life Technologies, A-21206). PE mouse anti-human CD133 antibody was obtained from Santa Cruz Biotechnology. Anti-M13 antibody was purchased from GE Healthcare (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). An *Escherichia coli* strain, Rosetta (DE3), was used as an expression host to produce PKCδ protein. Bacteria culture media, Bacto Tryptone, and Bacto Yeast Extract were purchased from Difco (Becton Dickinson, Franklin Lakes, NJ, USA). Restriction enzymes, Pfu DNA polymerase, T4 DNA ligase, and other cloning reagents were obtained from Takara Bio (Otsu, Shiga, Japan). An M13 peptide library screening kit (Ph.D. Phage Display Peptide Library Kit) was obtained from New England Biolabs (Ipswich, MA, USA). The protein kinase activity kit was purchased from Enzo Life Science (Farmingdale, NY, USA). $Ni^{+}$ Sepharose (GE Healthcare) was used to purify the target protein. Denatured protein was refolded using a Bio-Rad Gradient Econo pump purifier (Bio-Rad, Hercules, CA, USA). All other chemicals used in this study, including isopropyl-1-thiogalactopyranoside (IPTG), were obtained from Sigma (St. Louis, MO, USA). Substrate Reagent Pack (DY999) was purchased from R&D Systems (Minneapolis, MN, USA).

### *Methods*

#### *Gene cloning and protein purification*

The gene encoding the PKCδ catalytic domain was extracted from the U87 glioma cell line. Based on the cDNA from glioma cells, polymerase chain reactions (PCRs) were carried out using specific primers with the following sequences: 5′-CCT AGG ACC TAC GGC AAG ATC TGG GAG GGC-3′ and 5′-CTT AAG TAG AAG GTC CTC CAC GAG CTT AAA -3' (underlined nucleotide sequences represent the linkers of BamH1 and EcoRI, respectively). The PCR was performed with the following parameters: 95 °C for 3 min, 25 cycles at 94 °C for 2 min, 70 °C for 2 min, 72 °C for 2 min, and a final 10-min extension at 72 °C. The PCR product was transformed to Rosetta (DE3), and the cells were induced to express the protein by the addition of 0.5 mM IPTG at 18 °C overnight.

For protein purification, the cell pellet was dissolved in a resuspension buffer (20 mM Tris, pH 8.0). After a 30-s sonication step, the mixture was centrifuged at 14,000 rpm for 15 min twice. The cell pellet was resuspended using an isolation buffer (20 mM Tris, 400 mM NaCl, and 2% Triton X-100, pH 8.0) and centrifuged at 14,000 rpm for 15 min with a 30-s sonication step. The isolated pellet was resuspended using a binding buffer (6 M guanidine hydrochloride, 20 mM Tris, 400 mM NaCl, 5 mM imidazole, and 1 mM β-mercaptoethanol, pH 8.0) to denature the protein and then was incubated for 1 h at room temperature. After incubation, the solution was sonicated for 30 s and centrifuged at 14,000 rpm for 1 h at 4 °C. The supernatant was collected and loaded on an $Ni^{+}$ Sepharose affinity chromatography column using a Bio-Rad Gradient Econo pump purification system. The denatured protein was renatured using a refolding buffer with a gradient. Final products were eluted using an elution buffer, and the resulting eluted sample was loaded on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. The purified protein was dialyzed by incubating overnight in a semipermeable membrane with dialysis buffer at 4 °C. The final product was concentrated using a 10-kDa cutoff membrane (Ultrafiltration disc, Merck Millipore, USA).

The purified protein concentration was determined by the Bradford method with modifications to the protocol supplied by the manufacturer (Bio-Rad). The optical density was measure by spectrophotometer (Optizen 2120 UV), and the value for optical density was calculated by Lambert–Beer's law.

#### *Kinase assay*

To analyze the folding of the recombinant protein in vitro, we assayed the activity of the protein using a kinase assay kit (Enzo Life Science) [28]. Briefly, we soaked wells of the PKC substrate microtiter plate with a 50-μl kinase assay dilution buffer at room temperature for 10 min. We then added 2 μg of protein (cd-PKCδ, active PKC, bovine serum albumin [BSA]) to the well in addition to 20 μg of ATP. The mixture was incubated for 3 h at 37 °C. The reaction was stopped with an aspiration of the mixture. The phosphate-specific substrate antibody was added to each well and incubated for 1 h at room temperature. The well was washed 10 times with a washing buffer, and we then added the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. After a 2-h incubation, the well was washed 10 times and the tetramethylbenzidine (TMB) substrate was added. After a 30-min reaction time, the reaction was terminated with an acidic solution and the well contents were measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

#### *Plate binding assay*

To analyze the immobilized efficiency of the target protein on the plate, we measured the binding signal using the enzyme-linked