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Chemical Synthesis of a PKC C1b Domain by a Peptide Ligation Method and Expression of the Protein in E. coli and Their Application

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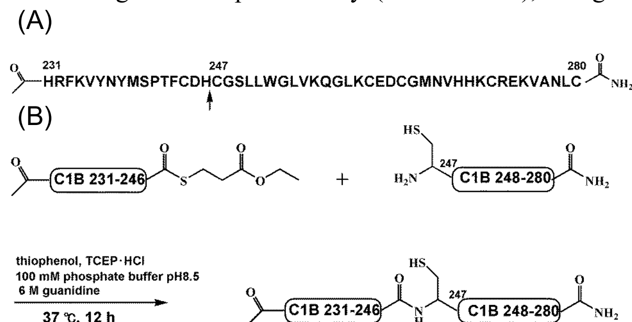
Introduction

Protein kinase C, PKC, is a family of enzymes for phosphorylation, which is specific for Ser and Thr residues. PKC family comprises at least 11 isozymes, which play fundamental roles in signaling pathways that regulate cell cycle progression, differentiation, and apoptosis. PKC activity is regulated by phosphorylation, by calcium, and by association with phospholipids and a physiological ligand, diacylglycerol (DAG). PKC has also been proven to be involved in problematic diseases, such as cancer and Alzheimer's diseases. Thus, it has been established as an important therapeutic target. Marquez's group has developed conformationally constrained analogs of DAG based on gamma-lactone templates [1,2]. In this study, we synthesized a PKCδ C1b domain, which comprises a binding pocket of DAG, by a native chemical ligation method. This domain was also expressed in *E. coli* to study structural analysis of its complex with the above DAG analogs.

Results and Discussion

<u>*Chemical synthesis of PKCδ C1b domain.*</u>

In the synthesis of a mouse PKCδ C1b domain (50 amino acid residues), a native chemical ligation method was utilized. In this case, an unprotected peptide α-carboxythioester (231-246; PKCδ-N) was reacted with another peptide containing an N-terminal cysteine residue (247-280; PKCδ-C) (Scheme 1). PKCδ-N and PKCδ-C were synthesized by Fmoc-solid phase peptide synthesis on a Rink amide resin and on a chlorotrityl resin, respectively. The ligated peptides were purified with HPLC and characteristed by electro-spray-ionization time-of-flight mass spectrometry (ESI-TOFMS), using a DALTONICS(BRUKER): PKCδ-N [MH$^+$] calcd 2203.5, observed 2203.2; PKCδ-C [MH$^+$] calcd 3772.5, observed 3771.19. The purified peptides were lyophilized and dissolved in buffer in advance of the ligation reaction. The thioester peptide (PKCδ-N: 2.9 mg, 1.3 μmol), N-terminal Cys peptide (PKCδ-C: 4.9 mg, 1.3 μmol) and TCEP·HCl (3 mg, 13 μmol) were dissolved in 1.5 mL of 6 M Gn·HCl, 0.1 M sodium phosphate at pH 8.5, and 5% thiophenol. After incubation at 37 ºC under N_2 condition, the reaction mixture was analyzed by reversed phase HPLC (column: COSMOSIL 5C_{18} AR-2, 4.6×250mm). Charts (A)-(C) in Fig. 1 show the progress of the ligation reaction at 0, 9, and 20 hours' incubation, respectively. The eluent was monitored at 220 nm. Labeled peaks 1-3 were identified by ESI-TOFMS; 1, PKCδ-N; 2, PCKδ-C; 3, PKCδ [MH$^+$] calcd 5841.0, observed 5842.1. Although the PKCδ C1b has six cysteine residues, this peptide was synthesized more efficiently using a ligation technique compared to a stepwise condensation method.



Scheme 1. (A)Peptide sequence of PKCδ C1b domain. (B)Scheme of native chemical ligation of PKCδ C1b domain.

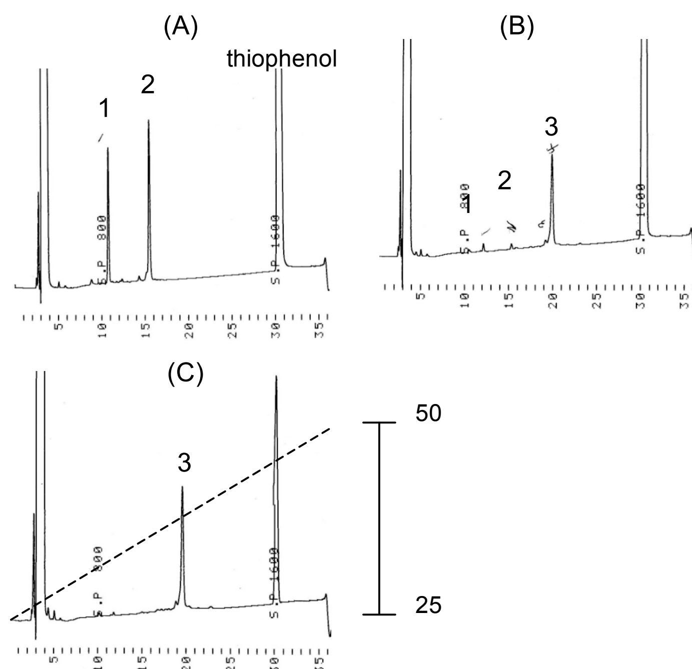


Fig. 1. Analytical HPLC data for the reaction mixture. The dashed line in (C) shows linear gradient of acetonitrile [%]. Peaks 1-3 indicate the elution of PKCδ-N, PKCδ-C, and a ligation product derived from both peptides (PKCδ), respectively.

Protein expression and purification of PKCδ C1b domain.

PKCδ C1b domain was expressed in *E. coli* as thrombin-cleavable glutathione S-transferase (GST) fusion protein containing N- and C-terminal extensions from the sequence (231-280); the entire protein contains 65 amino acid residues (Fig.2) [3]. DNA fragment coding PKCδ C1b domain was inserted as BamHI/EcoRI fragment into pGEX-2tk plasmid. The insert was confirmed by DNA sequencing. For protein expression, the plasmid was transformed with *E. coli* strain C41, which was derived from BL21(DE3). *E. coli* strain was maintained in Luria Bertania (LB) broth. For induction of protein expression under control of *lac* promoter, IPTG (0.3 mM) was added when OD600 reached to ~0.6. The bacterial culture was further incubated for 3 hours at 37 ºC. After incubation, *E. coli* was collected by centrifuge at 4 ºC. The pellet was resuspended in phosphate buffer (pH 7.4). Crude protein mixture was extracted by sonication. After sonication, extracted solution was centrifuged and the supernatant was proceeded to a purification step. The crude protein mixture was adsorbed on sepharose beads (glutathione sepharose beads: GE) and washed twice with cold phosphate buffer. Sequentially, thrombine solution was absorbed for GST-tag cleavage and incubated for overnight at room temperature. The cleaved PKCδ C1b domain was eluted in phosphate buffer. The peptide domain was further purified by gel filtration utilizing Superdex75 (Scheme 2). The phosphate buffer utilized at all purification steps contains 1 mM DTT (pH 7.4). The purity of peptide was confirmed by SDS-PAGE. Results of electrophoresis showed the purity of PKCδ C1b domain is more than 90% of total protein.

GS: thrombin cleavage site
RRASV: spacer
GS: delived from *Bam*H I site
GSRRASVGSHRFKVYNYMSPTFCDHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLC
mouse PKC δ C1b domain

Fig. 2. (A) Sequence of PKCδ C1b domain expressed as recombinant protein. (B) Scheme for purification of GST-fused PKCδ C1b domain.

Structure of recombinant PKCδ C1b domain.

The structure of PKCδ C1b domain expressed in *E. coli* was assessed by circular dichroism (CD) spectra (Fig. 3). The CD spectrum was recorded on a Jasco J-720 spectropolarimeter in a 0.1 cm path length cell under a nitrogen atmosphere at 25 ºC. The spectrum indicates that PKCδ C1b domain obtained as recombinant GST-fusion protein takes a similar folding structure as previously shown [4].

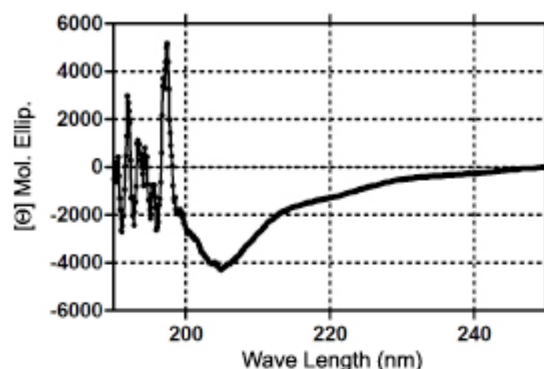


Fig. 3. CD spectrum of PKCδ C1b domain.

In summary, a PKCδ C1b domain, involving a binding pocket of DAG, was successfully synthesized by a native chemical ligation method. This protein was also expressed in *E. coli* as a recombinant thrombin-cleavable GST-fusion protein. Structural analysis of its complex with the above DAG analogs is in progress using this expressed protein. Fluorescent PKC mutants are also being prepared to develop biosensors of PKC ligands, which are useful for establishment of a new binding assay system.

Acknowledgments

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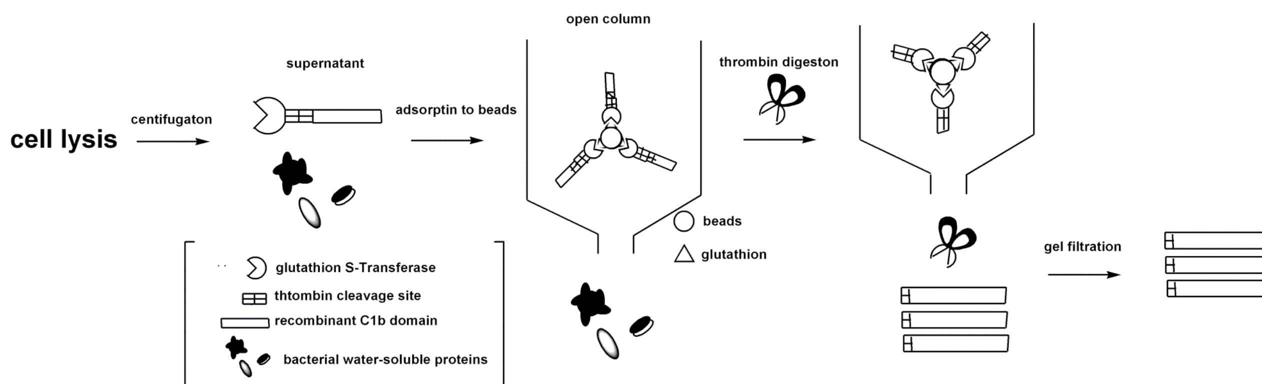

Scheme 2. Purification procedure of recombinant PKCδ C1b domain.