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Fluorescent-responsive Synthetic C1b Domains of Protein Kinase C δ as Reporters of Specific High Affinity Ligand Binding

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Abstract

Protein kinase C (PKC) is a critical cell signaling pathway involved in many disorders such as cancer and Alzheimer-type dementia. To date, evaluation of PKC ligand binding affinity has been performed by competitive studies against radiolabelled probes that are problematic for high-throughput screening. In the present study, we have developed a fluorescent based binding assay system for identifying ligands that target the PKC ligand binding domain (C1 domain). An environmentally sensitive fluorescent dye (solvatochromic fluorophore), which has been used in multiple applications to assess protein-binding interactions, was inserted in proximity to the binding pocket of a novel PKC δ C1b domain. These resultant fluorescent-labeled δ C1b domain analogues underwent a significant change in fluorescent intensity upon ligand binding, and we further demonstrate that the fluorescent δ C1b domain analogues can be used to evaluate ligand binding affinity.

Keywords

environmentally sensitive fluorescent dye; fluorescent-labeling; ligand binding; protein kinase C C1b domain

Introduction

Protein kinase C (PKC) is a family of serine/threonine protein kinases comprising 11 isozymes divided into three subclasses, termed conventional (α , $\beta_{I/II}$, γ), novel (δ , ϵ , η , θ), and atypical (ζ , λ , ι). Their classification is based on their essential structures and affinities for regulatory factors such as diacylglycerol (DAG) and calcium that bind to the C1 and C2 domains, respectively, of PKC. PKC plays a pivotal role in physiological responses to growth factors, oxidative stress, and tumor promoters (phorbol esters). These responses regulate numerous cellular processes (1,2), including proliferation (3), differentiation (4), migration (5), and apoptosis (6,7). The extensive involvement of PKC in both normal physiology and in numerous disorders has caused PKC to emerge an important therapeutic target (8–10). Since PKC activation is regulated through the binding of ligands to its C1 domains, development of useful ligands targeted to the C1 domains has been of intense

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interest for medicinal chemists. Various synthetic PKC ligands based on γ -lactone templates have been developed and evaluated (11–13). On the other hand, since structures of many of the potent, naturally occurring PKC ligands such as the phorbol esters are highly complex, it has been difficult to extensively probe their structure-activity relationships. Much opportunity therefore remains for the development of ligands optimized for isozyme selectivity or other properties. Fluorescent-based methods possess many advantages for high-throughput screening. Especially, utilization of environmentally-sensitive fluorophores suitable for high throughput techniques because washing-steps are not generally required. Fluorophores often respond to the environmental changes in hydrophobic/hydrophilic states associated with the conformational changes of proteins accompanying ligand binding. Several screening methods based on fluorescent-modified peptides, e.g. an IP₃ sensor, have been developed to date (14–17). In this study, fluorescent-labeled C1b domains of PKC δ utilizing a solvatochromic dye as a sensor of ligand binding were designed and synthesized as efficient screening tools to evaluate ligand binding and to explore novel PKC pharmacophores.

Experimental Procedures

General Methods

For chromatography, Wakogel C-200 (Wako Pure Chemical Industries, Ltd.) was employed. ¹H-NMR and ¹³C-NMR spectra were recorded using a Bruker ULTRASHIELD™ PLUS AVANCE 400 spectrometer. Relative chemical shifts were reported in δ (ppm) in DMSO-*d*₆ or in CDCl₃ with tetramethylsilane as an internal standard. Low- and high-resolution mass spectra were recorded on a JMS-T1000LC AccuTOF and Bruker Daltonics microTOF-2focus. RP-HPLC was performed with linear gradients of acetonitrile and H₂O containing 0.1% (v/v) TFA (column: COSMOSIL₅C₁₈ AR-II (4.6 $\times$ 250 mm) for analytical runs, COSMOSIL₅C₁₈ AR-II (20 $\times$ 250 mm) for preparative runs. UV absorbance spectra were recorded on a JASCO V-650 spectrophotometer using a 1.0 cm path length quartz cuvette. Fluorescent spectra were recorded on a JASCO FP-6600 spectrofluorometer using a 1.0 cm path length quartz cuvette. Measurements of fluorescent intensity on 96-well plates were performed on a Wallac ARVO MX (Perkin Elmer).

Peptide Synthesis

The protected peptide of δ C1b(247-281) was manually constructed on a Novasyn TGR resin (0.25 mmol/g) by standard Fmoc-based solid phase peptide synthesis (SPPS). Fmoc-protected amino acid derivatives (5 equiv) were successively condensed using 1, 3-diisopropylcarbodiimide (DIPCI) (5 equiv) in the presence of 1-hydroxybenzotriazole-H₂O (HOBt-H₂O) (5 equiv) in DMF (2mL) (90 min treatment). The following side-chain protecting groups were used; Boc for Lys, Pbf for Arg, OBU^t for Asp, Trt for Asn, Cys, and His, Bu^t for Ser, Thr, and Tyr. The Fmoc group was deprotected with 20% (v/v) piperidine in DMF (2 mL) for 15 min. The resulting protected peptide was cleaved from the resin and deprotected with TFA–thioanisole–*m*-cresol–triisopropylsilane (TIS) (89:7.5:2.5:1, v/v) (90 min treatment). Deprotected peptides were washed with cold Et₂O three times. The product was then purified by RP-HPLC. The protected peptides of dansyl-labeled δ C1b(221-246) were manually elongated on an Fmoc-His(Trt)-Trt(2-Cl)-resin (0.42 mmol/g) by Fmoc-based SPPS as in the synthesis of δ C1b(247-281). The following side-chain protecting groups were used; Boc for Lys, Pbf for Arg, OBU^t for Asp, Glu, Trt for Asn, Cys, Gln, and His, Bu^t for Ser, Thr, and Tyr. At the dansyl-labeled position, Fmoc-Lys(DnsGly)-OH was used. The resulting protected peptides were cleaved from the resin with trifluoroethanol (TFE)-AcOH-DCM (1:1:3, v/v) (2 h treatment), followed by thioesterification. Deprotection was performed as in the synthesis of δ C1b(247-281). The product was then purified by RP-

HPLC. Mass data and chemical yields of these peptides are described in Supporting Information.

Thioesterification

Thioesterification was performed with ethyl mercaptopropionate (20 equiv), HOBt·H₂O (10 equiv), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI)·HCl (10 equiv) in DMF (1 mL) (−20 °C, overnight). DMF was removed by evaporation, and the crude products were washed with H₂O.

Native Chemical Ligation

Dansyl-labeled δ C1b(221-246) (1.8 mg, 0.5 μ mol) and the δ C1b(247-281) (1.9 mg, 0.5 μ mol) were dissolved in 500 μ L of 100 mM phosphate buffer (pH 8.5) containing 6M guanidine hydrochloride (Gn·HCl) containing 2 mM EDTA and tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) (1.4 mg, 5 μ mol). Thiophenol (15 mL, 3%) was then added to the mixture. The ligation reaction was performed at 37 °C under an N₂ atmosphere. Progress of the ligation reaction was monitored by RP-HPLC (gradient; 25–45% of acetonitrile/0.1% TFA against H₂O/0.1% TFA). The product was subjected to gel filtration with Sephadex G-10 and then purified by RP-HPLC. Mass data of these peptides are described in Supporting Information.

Folding of δ C1b Domains

Purified peptides were dissolved in 50 mM Tris·HCl (pH 7.4) with 5 mM DTT, incubated for 15 min at 30 °C, and then stored at −20 °C with 20% glycerol. The peptide solution was dialyzed against 50 mM Tris·HCl (pH 7.4) containing 150 mM NaCl, 1 mM DTT, and 0.1 mM ZnCl₂ using a Slide-A-Lyzer Dialysis Cassette[®] 2,000 MWCO (Thermo Scientific).

[³H]PDBu Binding Assay

[³H]PDBu binding to the δ C1b domains was measured using the polyethylene glycol precipitation assay as described previously (18,19) with minor modifications. To determine the dissociation constants (K_d) and numbers of binding sites (B_{max}) for the dansyl-labeled δ C1b domains, saturation curves with increasing concentrations of [³H]PDBu were obtained in triplicate. Two-hundred and fifty μ L of the assay mixture contained 50 mM Tris·HCl (pH 7.4), 1 mM ethylenedis(oxyethylenenitrilo)tetraacetic acid (EGTA), 0.1 mg/mL phosphatidylserine, 5 mg/mL bovine immunoglobulin G, variable concentrations of [³H]PDBu and, for those tubes used to determine non-specific binding, an excess of non-radioactive PDBu. After addition of peptides stored in 0.015% Triton X-100, binding was carried out at 18 °C for 10 min. Samples were incubated on ice for 10 min. Two-hundred μ L of 35% polyethylene glycol in 50 mM Tris·HCl (pH 7.4) was added, and the samples were incubated on ice for an additional 10 min. The tubes were centrifuged at 4 °C (12,200 rpm, 15 min), and a 100 μ L aliquot of each supernatant was then transferred to a scintillation vial for determination of the amount of the free [³H]PDBu. After the remaining supernatant was aspirated off, the bottom of each centrifuge tube was cut off just above the pellet and transferred to a scintillation vial for the determination of the amount of the total bound [³H]PDBu. Dissociation constants (K_d) were calculated by Scatchard analysis.

CD Measurement

CD spectra were recorded on a JASCO J-720 spectropolarimeter at 25 °C. The measurements were performed at 0.1 nm spectral resolution using a 0.5 cm path length quartz cuvette. Each spectrum represents the average of 40 scans, and the scan rate was 100 nm/min. Measurements were performed in a Tris·HCl (pH 7.4) buffer containing 1 mM DTT and 150 mM NaCl.

Fluorescence Measurement

Ligand titration was performed in 1 mL of dialysis buffer containing 0.5 μ M dialyzed dansyl-labeled δ C1b domain and 5 μ g/mL phosphatidylserine at 25 °C. After each addition of ligands, the mixture was incubated at the same temperature for 10 min. Fluorescent emission spectra (λ_{ex} = 330 nm, slit width: 20 nm for excitation; 40 nm for emission) were obtained throughout the addition of ligands. For experiments using 96-cell plates, Dansyl-labeled δ C1b domain solution (dialysis buffer containing 0.5 μ M dialyzed peptides and 5 μ g/mL phosphatidylserine) was prepared and incubated for 10 min at room temperature. Fluorescence of dansyl-labeled δ C1b domain was measured using an excitation filter of 355 nm (half-width: 40 nm) and an emission filter of 460 nm (half-width: 25 nm), respectively.

Molecular Modelling

Molecular modelling was performed using SYBYL 7.1 (Tripos Inc., St. Louis, MO). Predictive models of dansyl-labeled δ C1b domain analogues were built by substitution of Lys(Dns-Gly) for residues Tyr238, Ser240 or Thr242 that were contained in the crystal structure of δ C1b domain (PDB; 1PTR) (20). Energy-minimization was performed on the Lys(dansyl-Gly) moiety using the Tripos force field and Gasteiger-Huckel charge parameters.

Supporting Information Available: Detailed materials and methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Results and Discussion

Design of Fluorescent-labeled PKC δ C1b Domains

Residues 221-281 of PKC δ form the C1b domain represent the starting sequence we used for modification. To identify the optimal amino acid position for fluorescent labeling, Tyr238, Ser240, and Thr242 were selected for evaluation. Our choice was based on the following rationale: First, these residues are located at the edge of the binding pocket of the C1b domain as shown by the structure of δ C1b-phorbol ester complex (20). Second, site-directed mutagenesis had shown that Ser240 was not necessary for the phorbol ester binding and that the δ C1b domain mutants T242G, T242S, and T242V had only minimal effects on the binding affinity of PDBu (2.1-, 1.1-, and 3.2-fold, respectively) (21). Third, Replacement of Tyr by Gly in position 238 reduced binding affinity of [3 H]PDBu by 60-fold, but maintained nanomolar affinity ($K_d = 48 \pm 3.0$ nM), which might be sufficient for the detection of PKC ligands (21). A chemically modified lysine was utilized for fluorescent labeling. For this study, a dansyl group was adopted because of its small molecular-size and larger Stokes shift compared to NBD (22). Since the flexibility of the dansyl moiety might contribute to its sensitivity to ligand binding to the δ C1b domain, glycine was incorporated between the ϵ -amino group of lysine and the dansyl moiety as a linker to construct Lys(Dns-Gly) (Scheme S1). Three δ C1b domain analogs, in which Lys(Dns-Gly) was substituted for either Tyr238, Ser240, or Thr242, were designed and designated as Y238K(DnsG), S240K(DnsG), and T242K(DnsG), respectively. Predictive structural models of the dansyl-labeled δ C1b domains were constructed based on the crystal structure of the δ C1b domain (PDB entry 1PTR) utilizing SYBYL (Figure 1). The models showed that the dansyl moieties of S240K(DnsG) and T242K(DnsG) are located outside the binding pocket, whereas the dansyl moiety of Y238K(DnsG) was located inside.

Synthesis of Dansyl-labeled δ C1b domain Analogs

Fmoc-protected Lys(dansyl-Gly) (NOH-016, 4) was synthesized as described in Scheme S1. δ C1b domain analogs were synthesized based on the standard Fmoc solid-phase peptide synthesis (SPPS) (23). For an efficient synthesis of dansyl-labeled δ C1b domain analogs, a

native chemical ligation (NCL) method was adopted (18, 24). Three N-terminal peptide fragments (dansyl-labeled δ C1b(221-246)) and a common C-terminal peptide fragment (δ C1b(247-281)) were separately synthesized. A purified δ C1b(247-281) and three dansyl-labeled δ C1b(221-246) fragments were condensed by an NCL method (Figure 2). The ligation reaction was performed with 0.8 mM of each peptide fragment in 100 mM phosphate buffer (pH 8.5) containing 6 M Gn-HCl, 2 mM EDTA and 3% thiophenol at 37 °C. The progress of the ligation reaction was monitored by HPLC (Supporting Information). The condensed δ C1b domains were identified by ESI-TOF-mass spectra (Table S1). The purified peptides were lyophilized and obtained as powders. Total yields of NCL were 38% for Y238K(DnsG), 48% for S240K(DnsG), and 41% for T242K(DnsG). Folding of the synthetic δ C1b domain was performed by dialysis against Zn^{2+} containing buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.1 mM ZnCl_2) at 4 °C.

Characterization of Synthetic δ C1b Analogs

The apparent [^3H]PDBu binding affinity of the dansyl-labeled δ C1b domains was evaluated by the method described previously (18,19). S240K(DnsG) and T242K(DnsG) showed K_d values comparable to that of the wild type. However, B_{max} values were 6% for S240K(DnsG) and 11% for T242K(DnsG) compared to the wild type (Table 1). The results indicate that dansyl-labeling might partly impair the efficiency of correct folding of the synthetic δ C1b domain. Y238K(DnsG) did not possess significant binding affinity for PDBu, consistent with the predictions from the modeling. We conclude that the dansyl-labeled δ C1b domains maintain potent binding activity and correct folding, at least for an appreciable proportion of the product, in those cases in which the dansyl group is located outside the binding pocket.

To estimate the influence of dansyl-labeling on folding of δ C1b domain, circular dichroism (CD) spectroscopy of S240K(DnsG) and T242K(DnsG) was performed for comparison before and after dialysis (Figure 3). Since the CD spectrum of the synthetic δ C1b(231-281) is similar with that of the recombinant δ C1b domain (18), Y238K(DnsG)- δ C1b(231-281) was used to evaluate effects of dansyl-labeling on folding of Y238K(DnsG). The addition of ZnCl_2 did not cause a significant change of CD spectra of Y238K(DnsG)- δ C1b(231-281). Thus, the introduction of a dansyl group into Tyr238 might interfere correct folding. The CD spectra of both of the dansyl-labeled δ C1b domains before dialysis showed broad minima around 205 nm, suggesting random coil structures (25). CD spectra of the dialyzed dansyl-labeled δ C1b domains exhibited decreases of negative cotton effects around 205 nm, which were similar to those of recombinant and synthetic δ C1b domains (18,25).

The apparent [^3H]PDBu binding affinities and CD spectra of the dansyl-labeled δ C1b domains, S240K(DnsG) and T242K(DnsG), demonstrated that insertion of dansyl groups at S240 and at T242 maintained correct folding and strong ligand binding affinity comparable to that of the wild type, although involving reduction of the stability of the domains.

Titration of Ligands to Fluorescent-labeled δ C1b Domain Analogs

To evaluate the fluorescent properties of dansyl-labeled δ C1b domain, fluorescent emission spectra were measured during ligand titration. As test ligands, the phorbol esters PDBu ($K_i = 0.72 \pm 0.06$ nM), phorbol 12-myristate 13-acetate (PMA) ($K_i = 0.14 \pm 0.04$ nM), and phorbol 12, 13-diacetate (PDA) ($K_i = 68.9 \pm 5.9$ nM), were employed. Values of K_i were determined by competitive binding assays using [^3H]PDBu (Supporting Information). Fluorescent titration experiments showed that the spectra of the dansyl-labeled δ C1b domain analogs changed according to the ligand concentration. S240K(DnsG) showed 1.3-, 1.8- and 1.2-fold increases in fluorescent intensity and blue shifts in the emission maxima upon additions of PDBu, PMA and PDA, respectively (Figure 4(A)–(C)). T242K(DnsG) showed

1.3-, 2.6- and 1.1-fold increases in fluorescent intensity and blue shifts in the emission maxima upon additions of PDBu, PMA and PDA, respectively (Figure 4(D)–(F)). The rank order of increases in fluorescent intensity upon addition of the above ligands to S240K(DnsG) and T242K(DnsG) thus corresponded to that of their K_i values.

Since dansyl-Gly showed stronger fluorescent intensity in a hydrophobic environment than in a hydrophilic environment (Figure S1), the increases in fluorescence intensity upon ligand titration of S240K(DnsG) and T242K(DnsG) suggested that the environment surrounding the dansyl moiety was changed to become more hydrophobic upon ligand binding (26–28). As predicted in the modeling study, the dansyl group was not located in the binding pocket, preventing binding. Even after ligand binding, the dansyl group will still be located outside the binding pocket. Thus, the binding of ligands to the binding pocket must make the environment surrounding the dansyl more hydrophobic, possibly due to interactions between dansyl and the long alkyl chains of the ligands. In addition, T242K(DnsG) showed stronger fluorescence intensity than did S240K(DnsG). This phenomenon corresponds to their relative $B_{\max}$ values observed in the [^3H]PDBu binding assay (Figure 1B and C, Table 1). A reason could be the hydrogen bonding network at the binding site of phorbol ester on the δC1b domain. The C20 hydroxy group of the phorbol ester accepts a hydrogen bond from the main-chain NH of T242 (20). Thus, the stronger fluorescent intensity of T242K(DnsG) could be due to a closer distance to the bound ligand. Furthermore, saturation of the increase in fluorescent intensity was observed when the ligand concentration reached 0.05–0.1 μM (10–20% of the peptide concentration) (Figure 5). This result is consistent with the $B_{\max}$ values of dansyl labeled δC1b : 6–11% of the wild type. The K_d values based on titration of ligands to the dansyl-labeled δC1b domains were evaluated (Table 2). As a control experiment, S240K(DnsG) dialyzed without Zn^{2+} did not show any significant increase in fluorescent intensity upon the addition of PDBu (Figure S2), suggesting that correct folding involving zinc-finger formation is critical for ligand binding. The detection limit might be the level of the fluorescent intensity of PDA. The results of titration experiments indicate that, upon ligand binding to correctly folded C1b domains, the site surrounding the ligand binding pocket becomes more hydrophobic. Even if some uncertainty remains about the relative contributions of the mechanisms leading to this change in hydrophobicity, the core finding is that ligand binding was detected by an increase in fluorescent intensity, which corresponds to binding affinity. The binding activity of ligands can be possibly evaluated by increases in fluorescent intensity on the consideration of LogP values.

S240K(DnsG) and T242K(DnsG) were employed in fluorescent experiments utilizing 96-well plates for initial assessment of their suitability to high-throughput screening. Plots of changes of fluorescent intensity against the ligand concentration showed dose-dependent curves similar to those in titration experiments (Figure 6). The results indicate that the present fluorescent-responsive C1b domains can be used for screening of novel PKC pharmacophores.

Conclusions

In this study, three kinds of dansyl-labeled δC1b domains, Y238K(DnsG), S240K(DnsG), and T242K(DnsG), were synthetically constructed in an efficient way by utilizing Fmoc-SPPS and an NCL method. The results of CD measurements and [^3H]PDBu binding assays indicated that the position of dansyl-labeling was critical for maintenance of native functions including proper folding. The ligand titration of dansyl-labeled δC1b showed that the change of fluorescent spectra corresponded to the K_i values of the ligands. Furthermore, S240K(DnsG) and T242K(DnsG) were utilized for measurements using a 96-well plate-based format, indicating that evaluation of ligand binding could be performed in a high-throughput fashion. The present fluorescent-responsive domains were successfully utilized

in vitro. However, through optimization of the stability of the fluorescent-labeled δ C1b domain, these domains might also be adapted for cell-based assays as efficient DAG sensors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structural models of dansyl-labeled δ C1b analogs. (A) Y238K(DnsG), (B) S240K(DnsG), and (C) T242K(DnsG). Space-filling models indicate phorbol esters and zinc atoms.

Figure 2.

Schematic representation of construction of dansyl-labeled δ C1b. i) 100 mM phosphate buffer (pH 8.5), 6 M Gn·HCl, 2 mM EDTA, TCEP·HCl, thiophenol, N_2 , 37 °C; ii) 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1mM DTT, 0.1 mM $ZnCl_2$, 4 °C.

Figure 3. Changes of CD spectra of dansyl-labeled δ C1b analogs, S240K(DnsG) (A) and T242K(DnsG) (B). Blue and black plots show profiles before and after dialysis against Zn^{2+} containing buffer, respectively.

Figure 4.

Fluorescent spectra obtained by ligand titration experiments for dansyl-labeled δ C1b analogs. Panels (A)–(C) show titrations of S240K(DnsG) against PDBu, PMA, and PDA, respectively. Panels (D)–(F) show titrations of T242K(DnsG) against PDBu, PMA, and PDA, respectively. Dansyl-labeled δ C1b analog: 0.5 μ M, buffer: 50 mM Tris·HCl (pH7.4), 150 mM NaCl, 1 mM DTT, 0.1 mM ZnCl₂, 5 μ g/mL phosphatidylserine, ligand: 0.02, 0.06, 0.1, 0.14, 0.18, 0.22, 0.26, 0.3 eq, $\lambda_{\text{ex}} = 330$ nm.

Figure 5. Plots of change of fluorescence intensity from titration experiments using S240K(DnsG) (A) and T242K(DnsG) (B). Dansyl-labeled δC1b analog: 0.5 μM , square: PMA, round: PDBu, triangle: PDA.

Figure 6. Plots of change of fluorescence intensity from 96-well plates-based titration experiments of S240K(DnsG) (A) and T242K(DnsG) (B). cross: PMA, circle: PDBu, square: DAG-lactone derivative.

Table 1Binding activity of the synthetic δ C1b analogs to [3 H]PDBu.

peptides	K_d (nM) ^a	B_{max} (pmol/mg) ^b
wild type	0.34 ± 0.08	38000
Y238K(DnsG)	n.d. ^c	11
S240K(DnsG)	0.18 ± 0.05	2100
T242K(DnsG)	0.35 ± 0.04	3700

^a Dissociation constant for the synthetic δ C1b binding to [3 H]PDBu. Mean $\pm$ SEM.^b Numbers of binding sites.^c not determined.

Table 2

K_d values of the fluorescent-labeled δ C1b domain analogs based on titration of ligands.

compound	S240K(DnsG)	T242K(DnsG)
	K_d (nM) ^a	K_d (nM) ^a
PMA	20.6	121.6
PDBu	5.83	110
PDA	Not convergent	Not fitting

^aDissociation constant for the fluorescent-labeled δ C1b domain analogs to each compound was calculated.