Screening for Protein Kinase C Ligands Using Fluorescence Resonance Energy Transfer

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Protein kinase C (PKC) is correlated with cell signaling pathways and also receives attention as a therapeutic target for cancer and Alzheimer-type dementia. The application of Förster/resonance energy transfer (FRET) phenomena to detect binding between proteins and small molecules, for example, PKC and its ligands, underlies a fluorescence-based assay method suitable for high-throughput screening. To accelerate studies on PKC functions in processing signals using small molecules and the development of drugs that target PKC, novel methods for the assessment of the PKC binding affinity of compounds are necessary. We previously developed solvatochromic fluorophore-based methods for that assessment. In this study, a novel method for a FRET-based PKC binding assay was developed and is expected to overcome the limitations of solvatochromic fluorophores.

Key words  protein kinase C; protein kinase C ligand; ligand screening; Förster resonance energy transfer; fluorescence resonance energy transfer

Förster/resonance energy transfer (FRET) is the transfer of excitation energy from a fluorescence donor to an acceptor. FRET efficiency and sensitivity depend on the distance between the donor and the acceptor. The FRET phenomenon can be applied as a spectroscopic measure and a probe of conformational change of macro-biomolecules and a FRET-mediated competitive assay can be used for studies of the binding between two molecules. Radioisotope-based methods are extremely sensitive and are widely used for ligand binding assays, but fluorescence-based assays, which are suitable for high-throughput screening are free of hazards associated with radioactivity. Previously, we described fluorescence-based ligand screening assays as an alternative to radioisotope assays.

Protein kinase C (PKC) isozymes, which are classified as Ser/Thr kinases, play a critical role in cellular signaling pathways related to proliferation, differentiation, and apoptosis. PKC has 11 isozymes which are classified into two subtypes: conventional PKC (cPKC; ε, δ, η, ζ, ι), novel PKC (nPKC; δ, ε, η, ι) and atypical PKC (aPKC; α, γ, λ, ι). The binding of diacylglycerols (DAG) to the C1 domain of PKC is an important step in an activation process except in the case of PKC and DAG. DAG containing an atypical C1 domain which fails to bind to DAG. Various synthetic PKC ligands targeting the C1 domain have been developed as chemical probes or drug candidates by fluorescent dyes such as 6-methoxynaphthalene (6MN) derivatives.


definitions

- **FRET**: Fluorescence Resonance Energy Transfer
- **PKC**: Protein Kinase C
- **DAG**: Diacylglycerol
- **cPKC**: Conventional Protein Kinase C
- **nPKC**: Novel Protein Kinase C
- **aPKC**: Atypical Protein Kinase C

Results and Discussion

**Design and Synthesis of FRET-Donor and FRET-Acceptor Molecules**  
Diethylaminocoumarin (DEAC) (λ<sub>ex</sub>=380nm, λ<sub>em</sub>=460nm, in MeOH) and fluorescein (λ<sub>ex</sub>=497nm, λ<sub>em</sub>=521nm, in buffer pH 7.4) were used in this study as the FRET donor and the FRET acceptor fluorescent dyes, respectively. Based on our previous reports, DAG-γ-lactones, whose structure–activity relationships (SAR), have been studied in detail, were adopted as a template for FRET donor molecules. We have observed that DAG-γ-lactones with fluorescent dyes such as 6-methoxynaphthalene (6MN) derivatives which contain two aromatic rings at the α-alkylidene position (sn-2), possess significant binding affinity for the PKC C1b domain. Furthermore, DEAC is more sensitive to

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solvents than 6-methoxynaphthalene or dansyl derivatives. In the previous report, a DAG-lactone labeled by DEAC at the acyl position (sn-1) showed a 2.6-fold higher $K_i$ value (93.3 nM) than a DAG-lactone labeled by 6MN at the $\alpha$-alkylidene position (sn-2) (35.9 nM). In this study, a DAG-lactone containing DEAC at the $\alpha$-alkylidene position was designed to improve a $K_i$ value. Initially, a DAG-$\gamma$-lactone template was prepared as reported previously. Oxidation of 7-diethylamino-4-methylycoumarin with selenium dioxide gave DEAC possessing an aldehyde group at the C4 position, and this was followed by an aldol condensation with the lactone $\mathcal{L}$. Deprotection of tert-butyldiphenylsilyl (TBDPS) and finally, monoacylation of the resulting diol gave sn-2 DEAC-type DAG-lactone $\mathcal{C}$ (E isomer, racemic) (Chart 1). DAG-$\gamma$-lactone derivatives have been used as racemic compounds.

The C1b domain, residues 231 to 281 of PKC$\delta$, was used as an acceptor molecule. Val235 was selected as the position to be labeled with fluorescein based on the following rationale: Since Val235 is located in the solvent accessible area, it is presumed that the labeled fluorescein would not disturb folding of the C1b domain (PDB ID: 1PTR). The distance of several dozen nanometers between donor and acceptor transforms FRET from a donor fluorescent dye to an acceptor fluorescent dye and consequently, use of the FRET phenomenon does not require introduction of an acceptor fluorescent dye precisely into the edge of the binding pocket. Accordingly, the fluorescein-labeled C1b domain (Fi-$\delta$C1b) 12 was synthesized by Fmoc solid-phase peptide synthesis as follows. Specifically, condensation with Fmoc-Nle($\varepsilon$-azide)-OH, followed by cycloaddition of alkyne suspending fluorescein to the azide at the $\varepsilon$-position of the Nle residue, and subsequent native chemical ligation generated Fi-$\delta$C1b (12) (Chart 2).

**Evaluation of sn-2 DEAC-Type DAG-Lactone and Synthetic $\delta$C1b by the Classical RI Assay** The wild type of $\delta$C1b(231–281) was synthesized as described previously. The $K_d$ value of the synthetic $\delta$C1b domain and the $K_i$ value of sn-2 DEAC-type DAG-lactone were determined by the poly(ethylene glycol) precipitation assay. The RI assay showed the Fi-$\delta$C1b domain to possess a $K_d$ value comparable to that of the wild-type, whereas the $B_{max}$ value of Fi-$\delta$C1b was four-fold smaller than that of the wild-type (Table 1). Such significant decrease of $B_{max}$ indicated the fluorescent moiety could partially impair the efficiency of folding as described previously: The $B_{max}$ value of C1b for the solvatochromic assay is less than one-tenth. In terms of the efficiency of folding, Fi-$\delta$C1b is more suitable than C1b for the solvatochromic assay. The sn-2 DEAC-type DAG-lactone showed modest affinity for the $\delta$C1b domain ($K_i$=182±18 nM, Table 2). To screen ligand candidates, a competitive probe with high affinity might not be suitable and the sn-2 DEAC-type DAG-lactone could be useful for the development of new screening methods.

**FRET Experiments** When the sn-2 DEAC-type DAG-lactone binds to Fi-$\delta$C1b, the distance between the donor fluorescent dye (DEAC) and the acceptor fluorescent dye (fluorescein) might be sufficient for energy transfer from the donor to the acceptor (Fig. 1). If a test compound binds to Fi-$\delta$C1b competitively, the distance between the donor and the acceptor might be too great to support FRET, and in such a case, the excitation of the donor fluorescence might cause emission of...
the donor fluorescence. In practice, the addition of Fl-δC1b to the assay solution containing sn-2 DEAC-type DAG-lactone resulted in an increase of fluorescent intensity of the acceptor (fluorescein). If this was followed by the addition of PDBu as a competitive ligand, a 14% decrease of fluorescent intensity (Figs. 3A–C) was observed. Since the decrease of fluorescent intensity was nearly saturated at 2µM PDBu, the PDBu titration did not show a concentration-dependent manner (Fig. 3C).

In Fig. 3B, the decrease of fluorescent intensity of the donor observed upon the addition of PDBu is due to the influence of solvatochromism of the donor fluorescent dye (DEAC).

Since direct excitation of the acceptor was observed upon the titration of Fl-δC1b in the absence of the donor, sn-2 DEAC-type DAG-lactone (Fig. 3D), the increase in the acceptor fluorescent intensity based on the FRET phenomenon. The IC₅₀ values of test compounds can be calculated with the FRET assay in case serial diluted concentrations are used.

Conclusion
In this study, a FRET-based competitive assay has been developed. sn-2 DEAC-type DAG-lactone as a donor molecule and Fl-δC1b as an acceptor molecule were synthesized and it was confirmed that the sn-2 DEAC-type DAG-lactone probe possesses modest affinity for the δC1b domain and Fl-δC1b for the [3H]PDBu binding affinity. The addition of PDBu to the mixture of sn-2 DEAC-type DAG-lactone and Fl-δC1b caused a decrease of the acceptor fluorescent intensity upon the irradiation with the donor’s excitation wavelength. The FRET-based competitive assay could be useful for PKC ligand screening and applicable to other protein-small molecule interactions.

Experimental
General
For thin-layer chromatography (TLC), Merck 60F254 precoated silica gel plates were employed. Column chromatography was performed with Wakogel C-200 (Wako Pure Chemical Industries, Ltd.) and silica gel 60 n (Kanto Chemical Co., Inc.). ¹H-NMR (400 MHz) spectra were recorded using a Bruker Avance III 400 spectrometer and ¹H-NMR (500 MHz) and ¹3C-NMR (125 MHz) spectra were recorded using a Bruker Avance 500 spectrometer. Chemical shifts are reported in δ (ppm) relative to Me₄Si (in CDCl₃) as the internal standard. Low- and high-resolution mass spectra were recorded on a Bruker Daltonics micrOTOF-2focus. For RP-HPLC, Cosmostil 5C₁₈ AR-II column (4.6×250 mm for analytical runs, 20×250 mm for preparative runs, Nacalai Tesque, Inc., Kyoto, Japan) was employed with a linear gradient of CH₃CN and H₂O containing 0.1% (v/v) trifluoroacetic acid (TFA) at a flow rate of 1 cm³ min⁻¹ on a JASCO PU-2089 plus (JASCO Corporation, Ltd., Tokyo, Japan) for analytical runs or 10 cm³ min⁻¹ on a JASCO PU-2086 plus for preparative runs, and eluted products were detected by UV at 220 nm. UV-Vis absorbance spectra measurements were performed with a JASCO V-650 spectrophotometer. Fluorescent spectra were recorded on a JASCO FP-6600 spectrophotofluorometer equipped with 1.0 cm path length quartz cuvette.

4-((5,5-Bis(((tert-butyldiphenylsilyl)oxy)methyl)-2-oxodihydrofuran-3(2H)-ylidene)methyl)-7-(diethylamino)-2H-chromen-2-one (4) Lithium diisopropylamide (LDA) (260 µL, 0.52 mmol) was added to a solution of lactone 3 (253 mg, 0.4 mmol) in tetrahydrofuran (THF) (1.5 mL) at −78°C and the resulting mixture was stirred for 2 h at −78°C. The reaction mixture was quenched with saturated aqueous NH₄Cl. The aqueous layer was extracted three times with Et₂O. The
combined organic layer was washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. To a solution of the crude aldol product in CH₂Cl₂ were added MsCl (62 µL, 0.8 mmol) and Et₃N (223 µL, 1.6 mmol) at 0°C. The resulting mixture was stirred for 2 h then allowed to warm to room temperature. To the reaction mixture was then added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (300 µL, 2.0 mmol) at 0°C, and the resulting mixture was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure, followed by purification by silica gel column chromatography (hexane/EtOAc = 5 : 1) to give the enone 4 (E isomer, 155 mg, 46% yield for two steps) as an orange solid. The E isomer was exclusively obtained, which was based on tentative assignment 729): \( R_f \) 0.43 (hexane/EtOAc = 3 : 1); \( {^1}H\)-NMR (400 MHz, CDCl₃) \( \delta \): 7.43–7.31 (20H, m), 7.20 (1H, d, \( J=9.2\) Hz), 6.80 (1H, s), 6.49 (1H, d, \( J=2.4\) Hz), 6.29–6.26 (1H, m), 5.91 (1H, s), 3.77 (4H, s), 3.59 (2H, s), 3.35–3.30 (4H, m), 1.66–1.12 (6H, m), 0.94 (18H, s). \( {^{13}}C\)-NMR (125 MHz, CDCl₃) \( \delta \): 172.21, 161.81, 156.53, 151.93, 150.69, 135.49, 135.43, 132.85, 132.65, 132.29, 129.91, 127.80, 125.68, 109.15, 108.81, 107.26, 97.63, 89.88, 64.28, 44.63, 28.14, 26.63, 19.14, 12.39. Electrospray ionization-mass spectrometry (ESI-MS) \( m/z \): 850.3953 (Calcd for C₅₂H₆₀NO₆Si₂ [M+H]+: 850.3954).

\[ 4-((5,5-Bis(hydroxymethyl)-2-oxodihydrofuran-3(2H)-ylidene)methyl)-7-(diethylamino)-2H-chromen-2-one \] (5)

Tetra butylammonium fluoride (TBAF) (720 µL, 0.72 mmol) was added to a solution of the enone 4 (155 mg, 0.18 mmol) in THF (1 mL) at 0°C. The resulting mixture was stirred for 2 h then allowed to warm to room temperature. The reaction was quenched with H₂O. The aqueous layer was extracted three times with CHCl₃. The combined organic layer was washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (CHCl₃/MeOH = 30 : 1) gave diol 5 (14.7 mg, 63% yield) as an orange solid: \( R_f \) 0.0 (CHCl₃/MeOH = 20 : 1); \( {^1}H\)-NMR (400 MHz, CDCl₃) \( \delta \): 7.31 (1H, d, \( J=8.8\) Hz), 7.08 (1H, s), 6.58–6.55 (1H, m), 6.50 (1H, d, \( J=2.4\) Hz), 5.99 (1H, s), 3.89–3.80 (4H, m), 3.68 (2H, s), 3.44–3.38 (4H, m), 2.09–2.06 (2H, m), 1.22–1.19 (6H, m). \( {^{13}}C\)-NMR (125 MHz, CDCl₃) \( \delta \): 172.30, 162.35, 156.36, 152.07, 150.82, 150.52, 132.48, 125.41, 108.83, 108.59, 107.37, 97.67, 90.11, 63.34, 44.73, 27.67, 12.41. ESI-MS \( m/z \): 374.1596 (Calcd for C₂₀H₂₄NO₆ [M+H]: 374.1604).

\[ 4-((7-(Diethylamino)-2-oxo-2H-chromen-4-yl)-methylene)-2-(hydroxymethyl)-5-oxotetrahydrofuran-2-yl)methyl Decanoate \] (6)

Et₂N (20 µL, 0.15 mmol) and decanoyl chloride (10 µL, 0.05 mmol) were added to a solution of diol 5 (37 mg, 0.1 mmol) in THF (2 mL) at 0°C. The resulting mixture was stirred for 2 h then allowed to warm to room temperature. The reaction mixture was concentrated under reduced pressure.
pressure, followed by purification by silica gel column chromatography (CHCl₃/MeOH = 20 : 1) to give the decanoate 6 (E isomer, racemic, 14.7 mg, 28% yield) as an orange oil: \( R_f = 0.16 \) (CHCl₃/MeOH = 3 : 1); 1H-NMR (400 MHz, CDCl₃) \( \delta \): 7.30 (1H, d, \( J = 9.2 \) Hz), 7.02 (1H, s), 6.57–6.54 (1H, m), 6.51 (1H, d, \( J = 2.8 \) Hz), 5.97 (1H, s), 4.36–4.26 (2H, m), 3.77–3.75 (2H, m), 3.67 (2H, s), 3.41 (4H, dd, \( J = 6.8, 7.2 \) Hz), 2.28–2.23 (2H, m), 2.07–2.03 (1H, m), 1.26–1.19 (20H, m), 0.88 (3H, t, \( J = 6.8 \) Hz).

13C-NMR (125 MHz, CDCl₃) \( \delta \): 173.40, 171.55, 161.85, 156.45, 151.60, 150.80, 149.13, 133.02, 125.31, 108.80, 108.71, 107.22, 97.74, 87.80, 63.45, 62.87, 44.73, 33.82, 31.83, 29.37, 29.23, 27.63, 24.76, 22.64, 14.09, 12.41. ESI-MS \( m/z \): 528.2952 (Calcd for C₃₀H₄₂O₇N \([M+H]^+\): 528.2961).

2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-5(6)-(prop-2-yn-1-ylcarbamoyl)benzoic Acid 30) 1-Hydroxybenzotriazole monohydrate (HOBt·H₂O) (150 mg, 1.1 mmol), Et₃N (153 mL, 1.1 mmol) were added to a solution of propargylamine hydrochloride (100 mg, 1.1 mmol) in CH₂Cl₂ (10 mL), 5(6)-carboxyfluorescein (376 mg, 1.0 mmol) and 1-ethyl-(3-(3-dimethylamino)propyl)carbodiimide hydrochloride (EDCI) (210 mg, 1.1 mmol) at \(-8^\circ\)C, and the resulting mixture was stirred at 0°C for 1 h, and then at room temperature for 2 h. The reaction was quenched with H₂O. The aqueous layer was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over MgSO₄, filtrated and concentrated under reduced pressure to afford the crude alkyne. Purification by HPLC (27–36% MeCN/H₂O, 50 min) gave alkynyl fluorescein (mixture of 5-, 6-isomers) (120 mg, 29%) as an orange powder. ESI-MS \( m/z \): 414.0997 (Calcd for C₂₄H₁₆NO₆ \([M+H]^+\): 414.0978).

Preparation of Fmoc-His(Trt)-Trt(2-Cl)-Resin 2-Chlorotrityl chloride resin (1.2 mmol/g, 1.0 g) (Novabiochem) was treated with Fmoc-His(Trt)-OH (0.4 mmol, 245 mg) and DIPEA (1.6 mmol, 280 µL) in dry N,N-dimethylformamide (DMF) (6 mL) for 1 h. After washing with dry DMF, CH₂Cl₂, and Et₂O, the resin was dried in a vacuum desiccator. The loading was determined by measuring UV absorption at 301 nm of the pipericine-treated Fmoc-His(Trt)-Trt(2-Cl)-resin (0.26 mmol/g).

Peptide Synthesis All peptides were synthesized by Fmoc-based solid-phase peptide synthesis (SPPS). The following amino acid side-chain protecting groups were used; Boc for Lys, Pbf for Arg, OᵣBu for Asp and Glu, Trt for Asn, Cys, and His, 3'Bu for Ser, Thr, and Tyr. The coupling reactions were performed by shaking for 1.5–2 h a coupling reaction mixture contained Fmoc-protected amino acid and \( N,N',N''\)-tetramethyluronium hexafluorophosphate (HBTU)/DIPEA/HOBt·H₂O. A solution of 20% pipericine in DMF was used for deprotection of Fmoc groups, achieved by shaking the reaction mixture for 20 min. The δClb(247–281) fragment was manually constructed on a TGR resin (0.22 mmol/g, 0.68 g). Deprotection and cleavage from the resin was performed by treatment with TFA (10 mL),

Fig. 3. Changes of Fluorescent Spectra during Titration Experiments
(A) The titration of the acceptor molecule against 200 nM donor molecule. The concentration of the acceptor molecule was increased to 1120 nM (5.6 eq to the donor molecules) by titration. (B) The titration of PDBu against the assay solution containing 200 nM donor molecule and 1120 nM acceptor molecule. The concentration of PDBu was increased to 8 µM (40 eq to the donor molecule) by titration. (C) The plots of percent decrease of fluorescence intensity versus the concentration of PDBu at 522 nm. (D) The fluorescent spectra of acceptor molecules (133, 267, 533, 800, 1067 nm). The fluorescence intensity was increased with increasing concentration. The excitation wavelength was 380 nm.
m-cresol (0.25 mL), thioanisole (0.75 mL), 1,2-ethanediethiol (0.75 mL), H₂O (0.25 mL), and TIS (0.10 mL). Purification by isocratic RP-HPLC (30% isocratic of MeCN containing 0.1% TFA, vs. H₂O containing 0.1% TFA) gave 120 mg (21% yield) of the δC1b(247–281) fragment 11 as a white powder: ESI-MS m/z: Found 3828.3 (Calcd for C₁₆₁H₂₆₈N₅₁O₄₅S₆ [M+H]⁺: 3828.8). Retention time: 12.3 min with MeCN (30% isocratic).

The δC1b(231–246) and δC1b(231–246)V235Nle(α-azide) fragment was constructed on an Fmoc-His(Trt)-2-chlorotrityl resin 0.13 mmol scale using Fmoc-Nle(α-azide)-OH. Cleavage from the resin was performed by stirring for 2 h with TFE/AcOH/CH₃CN (1:1:3, v/v) vs./z/. The resulting protected peptide and HOBt·H₂O (10 eq) were dissolved in DMF, and ethyl 3-mercaptopropionate (20 eq) was added. The mixture was cooled to 0°C, and EDCI (10 eq) was added. The mixture was stirred for 5 h then allowed to reach room temperature. DMF was removed by evaporation, and the crude product was washed with H₂O. Deprotection was performed as in the synthesis of δC1b(247–281) without the addition of EDT. Preparative RP-HPLC (31% isocratic of MeCN) gave 8.8 mg (7% yield) of the δC1b(231–246)V235Nle(α-azide) thioester 8 as a white powder: ESI-MS m/z: Found 2259.1 (Calcd for C₁₆₁H₁₄₁N₃₂O₃₂S₆ [M+H]⁺: 2258.1). Retention time: 9.2 min with MeCN (27% isocratic).

Fluorescein Labeling by Click Reaction To a solution of δC1b(231–246)V235Nle(α-azide) (3 mg, 1.1 µmol) in DMF (120 µL) and H₂O (120 µL) was added 0.2 mM fluorescein alkyne in MeCN/H₂O 1:1 (55 µL, 1.1 µmol). After the addition of 0.1 mM CuSO₄ in H₂O (130 µL, 13 µmol) and 0.1 mM sodium ascorbate in H₂O (130 µL, 13 µmol), followed by degassing, the reaction mixture was stirred under an Ar atmosphere for 2 h at room temperature. Purification by RP-HPLC (gradient: 26–36% of MeCN, 60 min) gave 1.6 mg (53% yield) of the δC1b(231–246)V235Nle(α-Fl-Fl) (Fl-δC1b(231–281)) thioester 9 as a yellow powder. ESI-MS m/z: Found 2671.1 (Calcd for C₁₆₁H₁₇₃N₃₂O₅₂S₆ [M+H]⁺: 2671.1). Retention time: 14.2 min with MeCN (26–36%, 30 min).

Condensation of Peptide Fragments by Native Chemical Ligation The δC1b(231–246)V235Nle(α-Fl) containing the peptide thioester 9 (1.8 mg, 0.66 µmol) and δC1b(247–281) (2.8 mg, 0.60 µmol) were dissolved in 230 µL of 0.1 M phosphate buffer (pH 7.7) containing 6.0 M guanidine hydrochloride (Gn·HCl), 2.0 mM ethylenediaminetetraacetic acid (EDTA), TCEP-HCl (0.95 mg, 3.3 µmol) and 4-mercapto-phenylacetic acid (MPAA) (1.4 mg, 8.3 µmol). The reaction mixture was adjusted to approximately pH 7.8 with 8 M NaOH in H₂O. The ligation reaction was performed at 37°C for 5 h under an Ar atmosphere. Purification by RP-HPLC (gradient: 33–38% of MeCN, 60 min) gave 0.54 mg (13% yield) of δC1b(231–281)V235Nle(α-Fl) (Fl-δC1b(231–281)) 12 as a yellow powder. ESI-MS m/z: Found 6365.0 (Calcd for C₁₆₁H₁₄₁N₃₂O₅₂S₆ [M+H]⁺: 6363.9). Retention time: 19.0 min with MeCN (33–38%, 30 min).

Degeneration of Peptides A buffer (pH 7.4) containing 6 M Gn·HCl and 50 mM Tris·HCl, dithiothreitol (DTT) was made up to 50 mM, and then the peptide was dissolved in the solution at a concentration of approximately 30 µM. The peptide solution was incubated for 15 min at 30–37°C. The treated 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 3500 MWCO (Thermo Scientific) at 4°C. The dialysis buffer was changed three times. After dialysis the peptide solution was centrifuged at 15000 rpm for 15 min at 4°C, followed by the determination of concentration by UV absorption at 280 nm (ε: Tyr; 1280, Trp; 5690) or at 490 nm (ε: fluorescein; 76900).12,33

[3H]PDBu Binding Assay The dissociation constant (Kᵦ) of synthetic δC1b for [3H]PDBu binding and the inhibition constant (Kᵢ) of fluorescent-labeled DAG-lactone for binding of the δC1b domain were assessed by the poly(ethylene glycol) precipitation assay as described previously.1,2,7,26

FRET Experiment A phosphatidylserine (PS) solution was prepared as described below: 50 µL of 10 mg/mL PS in CHC₁₃ was transferred to a 2 mL micro tube and dried under a stream of N₂ gas. One milliliter of 50 mM Tris·HCl (pH 7.4) was added to the residue and the mixture was sonicated with a probe-type sonicator (5 s bursts × 3). The FRET assay solution (1 mL) containing 200 nM FRET donor molecule, 100 µg/mL PS, and 50 mM Tris·HCl (pH 7.4) was prepared with 200 µL of 0.5 mg/mL PS solution, 2 µL of 100 µM FRET donor stock in dimethyl sulfoxide (DMSO) and 800 µL of 50 mM Tris·HCl (pH 7.4). After addition of 13 µL of 10.5 µM FRET acceptor solution, the assay solution was mixed by pipetting followed by 5 min incubation in the dark. The fluorescent spectra were measured by excitation at 380 nm. The concentration of the FRET acceptor started at 140 nM and increased to 1120 nM. The FRET competitive assay was demonstrated using PDBu as a positive ligand. The competitive assay solution contains 200 nM FRET donor molecule, 100 µg/mL PS, and 50 mM Tris·HCl (pH 7.4). The concentration of PDBu started at 2 µM and increased to 8 µM.

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