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QUARTERLY

The ultraviolet studies on protein-lipid interaction of a protein kinase C-γ phorbol-binding domain*°

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Family of protein kinase C (PKC) isozymes play a key role in transducing a vast number of signals into the cells. The members of classical PKC family are activated by binding of various lipid ligands to one of the several cysteine-rich domains of the enzyme. Second cysteine-rich (Cys2) domain of PKC-γ was expressed in *Escherichia coli* as a fusion protein with glutathione-*S*-transferase (GST) using the cDNA sequence from rat brain. The Cys2 protein after cleavage from GST was purified to homogeneity using glutathione-agarose and Mono-S cation exchanger column. In order to investigate the interaction of lipids and calcium with Cys2 protein we used UV spectroscopy. The UV spectrum of Cys2 protein exhibited a maximum at 205 nm. Exposition of Cys2 protein to phosphatidylserine (PS) vesicles resulted in significant decrease in the absorbance in the 210 nm region. Changes in UV spectrum of Cys2 protein induced by phorbol 12,13-dibutyrate (PDB) were smaller than those induced by PS, and addition of PDB with PS had no effect on the PS induced changes in UV

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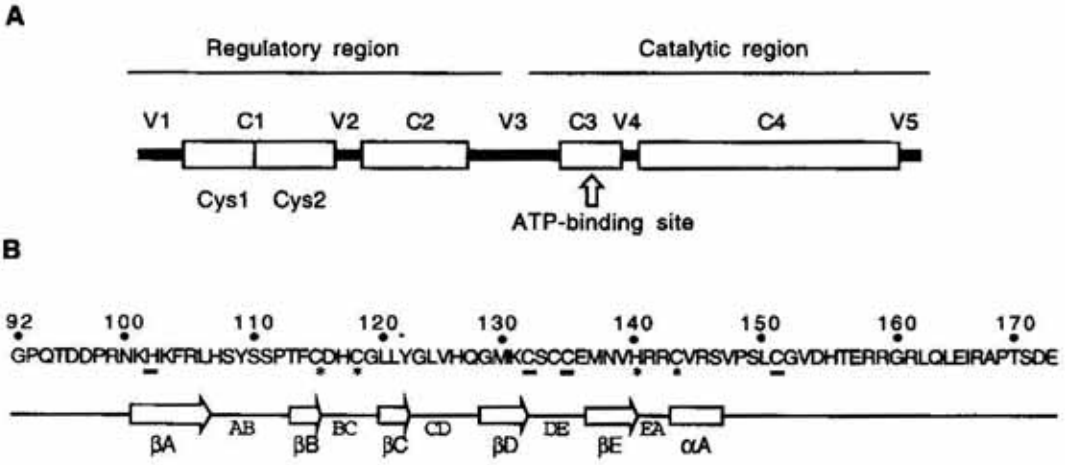
Abbreviations: PKC, protein kinase C; Cys2, second cysteine-rich region of PKC-γ; DAG, diacylglycerol; PDB, phorbol dibutyrate; GST, glutathione *S*-transferase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PBS, phosphate buffered saline; IPTG, β-D-thiogalactopyranoside.

spectrum of Cys2. Neither phosphatidylcholine (PC) nor phosphatidylethanolamine (PE) affected UV spectrum of Cys2 but in the presence of phosphatidylinositol 4,5-bisphosphate (PIP_2) or phosphatidylinositol 4-phosphate (PIP) vesicles some changes were observed. Calcium ions alone or in the presence of PS had no effect on the UV spectrum of Cys2 protein. These data indicate that PS comparing to PDB, interacts with a larger area of Cys2 protein, and that the binding sites for these two molecules are at least overlapping. The site of PIP and PIP_2 interaction with PKC-γ is distinct from that of phorbol ester binding site.

Protein kinase C-γ belongs to the group of conventional protein kinase C (PKC) isozymes. This class of kinases are regulated by Ca^{2+}, diacylglycerol (DAG) and phospholipids of which phosphatidylserine (PS) is indispensable [1–3]. The natural product phorbol and ingenol esters, potent tumor promoters, can substitute for diacylglycerol in activating PKC [1–3]. Cloning of PKCs revealed existence of four conserved domains within PKC [4]. The C1 domain in conventional PKCs contains a duplicated Cys-rich motif that forms the diacylglycerol/phorbol ester binding site (Scheme 1) [3]. The C2 domain contains the recognition site for acidic lipids and the Ca^{2+} binding site [5]. The C3 and C4 domains form the catalytic region of PKC with the binding site for ATP and the substrate [6]. Studies with the use of PKC-γ deletion mutants revealed two zinc-binding domains within the C1 region [7, 8]. These zinc-binding sites are located within cysteine-rich domains Cys1 and Cys2. Each of these two domains, expressed as recombinant proteins, bound two mole equivalents of zinc and demonstrated affinities for DAG and phorbols comparable to those of intact PKC protein [3, 8, 9]. Site-directed mutagenesis studies and an X-ray crystal structure of a PKC-δ Cys2/phorbol complex confirm that the Cys2 domain repre-



Scheme 1. A. Linear representation of the domains identified in the conventional protein kinase C (PKC) isozymes.

Variable (V) and constant (C) domains are indicated. Cys1 and Cys2 are cysteine-rich regions.

B. The sequence of Cys2 domain of rat brain PKC-γ.

The amino acids that make up the first (underlined), and second (*) zinc site are indicated. Below secondary structure elements of the Cys2 protein are shown. The NMR studies of PKC-γ Cys2 protein [12] showed that Cys2 has five β-strands (βA, βB, βC, βD, βE) and one α-helix (αA). There are three β-turns (BC, DE, EA) and two loops (CD, AB).

sents the domain responsible for DAG/phorbol binding and subsequent activation of PKC [10, 11]. Interaction of PKC-γ Cys2 domain with DAG and phorbol esters was also studied in solution by multidimensional heteronuclear NMR [12]. These studies provided an evidence that the top half of the Cys2 domain, including the phorbol site and one of the zinc sites, is capable of inserting into membranes.

Besides, DAG and phorbol esters, several negatively charged lipid effectors are also capable of partial PKC activation at relatively high concentrations of Ca^{2+}. In the presence of DAG and Ca^{2+} anionic phospholipids such as PS, phosphatidic acid (PA) and phosphatidyl-3-hydroxypropionate (PP) are sufficient to support the binding of PKC to the Triton X-100 mixed micelles, however, only PS activated PKC [13, 14]. Although PS activates PKC most efficiently, other phospholipids modulate the PS-dependent PKC activity. Phosphatidylethanolamine (PE) stimulated and phosphatidylcholine (PC) and sphingomyelin inhibited PKC [15]. Acidic phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol (PI) also activated PKC directly in a Ca^{2+}- and PS-dependent manner [16–18]. PIP_2 was also shown to inhibit phorbol esters binding to PKC [19]. The results cited above suggested that PIP_2 interacts with PKC at the DAG/phorbol ester binding site. Since, our previous study with the use of multidimensional heteronuclear NMR [12] provided evidence that Cys2 domain of PKC-γ, is capable of binding phorbol ester and other phospholipids we have extended investigation on PIP_2, PIP, PI, PE, and PC binding to this domain. Interaction of phorbol, phospholipids and calcium with purified to homogeneity recombinant Cys2 protein was examined by UV spectroscopy. Our results indicate that PE, PC and PI do not bind to Cys2 protein. Only very weak interaction of PIP_2 and PIP with Cys2 protein was detected. Thus, we conclude that PIP_2 activates PKC by mechanisms different from that of DAG/phorbol ester, and that the site of PIP_2 interaction with PKC is distinct from that of phorbol ester binding site.

Expression of Cys2 protein in *E. coli* and purification of this protein was previously briefly reported [12]. In the present paper a detailed procedure has been described with full documentation of individual purification step.

MATERIALS AND METHODS

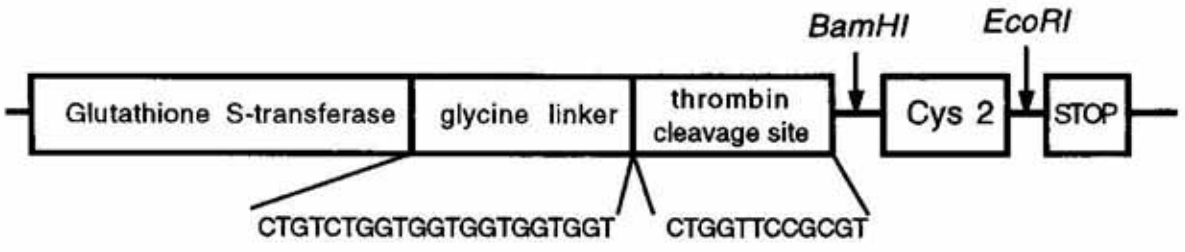
Reagents. Leupeptin, benzamidine, glutathione (reduced), β-mercaptoethanol, isopropyl β-D-thiogalactopyranoside, glutathione-agarose, phosphate buffered saline (PBS), carbenicillin, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol, 4α- and 4β-phorbol 12,13-dibutyrate were from Sigma-Aldrich Sp. z o.o. (Poznań, Poland). Phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, 1,2- and 1,3-dioctanoylglycerol were from Avanti Polar Lipids, Inc. (Birmingham, AL, U.S.A.). Pefabloc SC was from Boehringer Mannhein GmbH Biochemica (Mannhein, Germany). *Esccherichia coli* strain BL21(DE3) was from Novagen (Madison, WI, U.S.A.). Thrombin (bovine, plasminogen free) was from Calbiochem (San Diego, CA, U.S.A.). Mono S column was from Pharmacia Biotech Ges.m.b.H. (Wien, Austria). BioLogic System (FPLC) was from Bio-Rad Laboratories Ges.m.b.H. (Wien, Austria).

Protein expression. *E. coli* cells BL21(DE3) were transformed with pGEX-2TKG vector [20] with cloned cDNA for Cys2 domain (92–173) (Scheme 1) of rat brain PKC-γ. The vector contained GST gene linked to the cDNA of Cys2 domain through an extended thrombin cleavage site (Scheme 2). Cells were plated on plates containing ampicillin (50 μg/ml) and incubated overnight at 37°C. Single colony from a freshly streaked plate was grown overnight at 37°C in LB me-

dium (50 μg/ml carbenicillin). 1 mL of this culture was inoculated into 1 L of fresh LB medium and grown at 37°C with vigorous shaking until A_{600} reached 0.6. At this moment temperature was lowered to 23°C and cells were cultured until A_{600} reached 0.8. Expression was induced by adding IPTG to a final concentration of 1 mM. The cells were then shaken vigorously at room temperature for 12 h. At the end cells were harvested by centrifugation.

Preparation of cell extract. Cells from 1 L culture, either fresh or previously frozen, were harvested by centrifugation and suspended in 40 mL of ice cold buffer A (PBS, pH 7.4, 1 mM EDTA, 5 mM β-mercaptoethanol, 10 μg/mL leupeptin, 0.2 mM Pefabloc SC, 1 mM benzamidine). The cell suspension was then placed on powdered dry ice and sonicated using large probe at full power for six 1 min bursts with approximately 30 s intervals for cooling. The crude extract was diluted (1:1) with ice-cold buffer A and centrifuged at 50000 × *g* for 30 min (4°C). The obtained supernatant was supplemented with Triton X-100 (10%) to final concentration 1%.

Purification of Cys2 protein. All purification steps were performed at 4°C unless indicated. A column packed with 3 mL agarose-glutathione was equilibrated with 50 mL of buffer A supplemented with 1% Triton X-100. The supernatant of the crude extract was applied to the column at a flow rate of 50 mL/h. After the extract had been absorbed, the column was washed successively with 200 mL of buffer B (PBS, pH 7.4, 1 mM EDTA, 5 mM β-mercaptoethanol, 10 μg/mL leupeptin, 0.2 mM Pefabloc SC, 1 mM ATP, 10 mM $MgCl_2$, 1% Triton X-100) at a flow rate of 100 mL/h (not longer than 2–2.5 h) and then with 1 L of buffer C (PBS, pH 8.0, 2 mM β-mercaptoethanol) at a flow rate of 80 mL/h. Finally GST-Cys2 protein eluted with 30 mL of PBS, 20 mM Tris/HCl, 5 mM glutathione (reduced form) at a flow rate of 30 mL/h. Fractions of 1.2 mL were collected and those containing over 0.4 mg protein were combined and desalted on Sephadex G 25M equilibrated with PBS, pH 7.4. Desalted solution of GST-Cys2 was applied to the column packed with 2 mL glutathione-agarose. The column was washed with 30 mL of PBS, pH 7.4 at a flow rate of 30 mL/h at room temperature. Then the agarose with absorbed GST-Cys2 was incubated with 5 mL of PBS, pH 7.4, 2.5 mM $CaCl_2$, 40 U thrombin at room temperature for 3 h. Cleaved Cys2 protein was eluted with 10 mL of PBS, pH 7.4, at a flow rate of 10 mL/h. The solution of Cys2 protein was supplemented with dithiothreitol to 50 mM, and the solution was dialyzed to 2 L of 50 mM Tris/HCl buffer, pH 7.2, for 9 h (membrane molecular cutoff 8000). Dialyzed solution of Cys2 protein was applied to the Mono S (HR 5/5) column equilibrated with 50 mM phosphate buffer, pH 7.0. The column was washed with 5 mL of running buffer, then Cys2 protein was eluted with KCl gradient (0–1 M over 20 mL). Fractions of 0.5 mL were collected. Purity of each protein fraction eluted between 310–325 mM KCl was verified using 15% PAGE. Fractions containing pure Cys2 protein were combined and



Scheme 2. Cloning site in pGEX-2TKG vector.

The Cys2 was cloned between *Bam*HI and *Eco*RI site.

dialyzed to the required solution. Dialyzed solution was concentrated on Centricon-3 (Amicon) to 10 mg/mL.

UV spectroscopy. UV spectra were measured from 190 to 300 nm using a Philips PU 8720 UV/VIS scanning spectrophotometer. The measurements were performed at 25°C with 50 μg of Cys2 protein in 1 mL of 50 mM Hepes/NaOH, pH 7.4, 100 mM KCl with additions as indicated in the legends to the figures. Spectra were corrected for the absorbance of a solvent and lipids. The spectra represent an average of three digitized spectra from individual experiments. The spectral differences were reproducible over multiple experiments. An estimated error was ±5%.

Phospholipid vesicles preparation. Solution of the lipids to be studied were mixed and evaporated to dryness under nitrogen. The residue was sonicated under nitrogen for 10 min in the presence of 50 mM Hepes/NaOH, pH 7.4, 100 mM KCl. Phospholipid concentrations were determined after perchloric acid digestion [21] by measuring orthophosphate [22].

Binding to phospholipid vesicles. Binding experiments were performed according to the Harlan *et al.* [23]. Phospholipid vesicles (200 μg of total phospholipid) and Cys2 protein (typically 10 μg) were added to the buffer containing 50 mM Hepes/Na, pH 7.4, 100 mM KCl, 1 mM dithiothreitol, and 300 μM EGTA in a final volume of 0.15 mL. After incubation for 5 min at room temperature free and bound Cys2 protein were separated by centrifugation of the incubated mixture at 100000 × ***g*** for 30 min. Protein concentration of the supernatant (100 μL) was determined by the Bio-Rad Protein Microassay and compared to that of protein not exposed to phospholipids. In protein assay phospholipid blanks were run to correct for any background. Results are expressed as the Cys2 bound/Cys2 total (in the medium without centrifugation) ratio ×100. The recovery of phospholipid vesicles during centrifugation was 96–98% as determined with the vesicles containing [2-^{3}H]phosphatidylcholine.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate was performed according to Laemmli [24]. Protein bands were developed by Coomassie Brilliant Blue staining.

Protein was determined by the Bradford method [25] using bovine serum albumin as a standard, or by measuring the absorbance at 280 nm.

RESULTS

Expression and purification of Cys2 domain of protein kinase C-γ

The temperature used for expression of GST-Cys2 was crucial for optimum yield of soluble protein. Expression at 37°C for 3 h after induction produced 0.9–1.3 mg of GST-Cys2 protein from 1 L of culture eluted from glutathione-agarose. When the expression was induced in the cells growing at 23°C 15–20 mg of fusion protein was obtained from 1 L culture. The optimal yield of GST-Cys2 protein was obtained after 12 h of cell growth. Further elongation of the expression time at 23°C do not increased the yield of the protein (Fig. 1). Significant amount of the protein expressed at 23°C was soluble (recovered in the high-speed supernatant) (Fig. 1). The GST-Cys2 protein purified by affinity chromatography on glutathione-agarose resin was essentially pure; the protein showed very minor impurities only when the gels were grossly overloaded (Fig. 2). Glutathione *S*-transferase fusion proteins can be expressed using the vector pGEX-2T (Pharmacia). This vector introduced a thrombin cleavage site C-terminal to the GST protein. Obtained fusion protein can be readily purified using glutathione-agarose chromatography. Previously expressed GST-Cys2 protein with the use of pGEX-2T vector was poorly digestible by

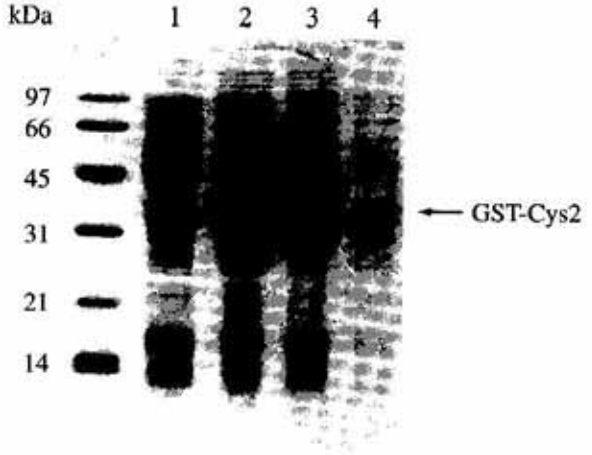


Figure 1. SDS/PAGE (12%) of GST-Cys2.

Lane 1, 50 μg of *E. coli* cells lysate (not induced); lane 2, 50 μg of lysate of *E. coli* cells induced for 12 h at 23°C; lane 3, 50 μg of lysate of *E. coli* cells induced for 18 h at 23°C; lane 4, 50 μg of high-speed supernatant of *E. coli* cells induced for 12 h at 23°C. The position of GST-Cys2 protein is indicated by arrow. On the left molecular mass markers are showed.

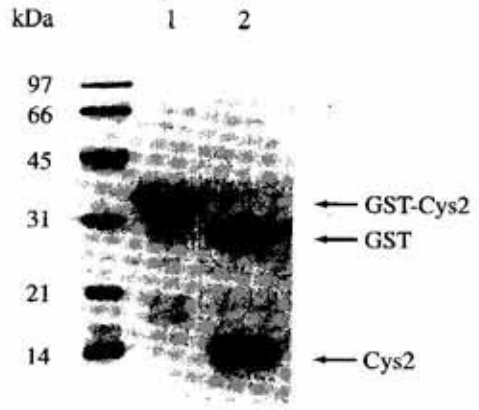


Figure 3. Thrombin cleavage of the GST-Cys2 fusion protein. 12% polyacrylamide/SDS gel.

The GST-Cys2 fusion protein (lane 1) was cleaved with thrombin as described under Materials and Methods. On lane 2 cleaved GST and Cys2 proteins are showed. On each lane (1 and 2) 20 μg of protein was loaded. On the left molecular mass markers were run.

thrombin [8, 9]. Here, we used a modified pGEX-2T vector namely pGEX-2TKG in which a glycine spacearm is placed N-terminal to the thrombin cleavage site (Scheme 2) [20]. Such modification dramatically improved the thrombin cleavage efficiency. Under condition used the Cys2 protein was completely cleaved from GST (Fig. 3). The major impurities at this point were β-mercaptoethanol adducts, which were hydrolysed during dialysis by addition of 50 mM dithiothreitol to the dialysis bag. The digested Cys2 protein was finally purified to homogeneity on Mono-S cation exchanger column (Fig. 4A). This step was necessary to separate properly zinc-coordinated Cys2 from unfolded and misfolded species. Purity of Cys2 protein eluted at about 320 mM KCl was confirmed by electrophoresis (Fig. 4B) and electrospray ionization mass spectrometry. Usually from 1 L of *E. coli* culture 2.5 mg of pure Cys2 protein was obtained.

UV spectroscopy

Changes in ultraviolet absorbance of proteins below 240 mn region are observed when

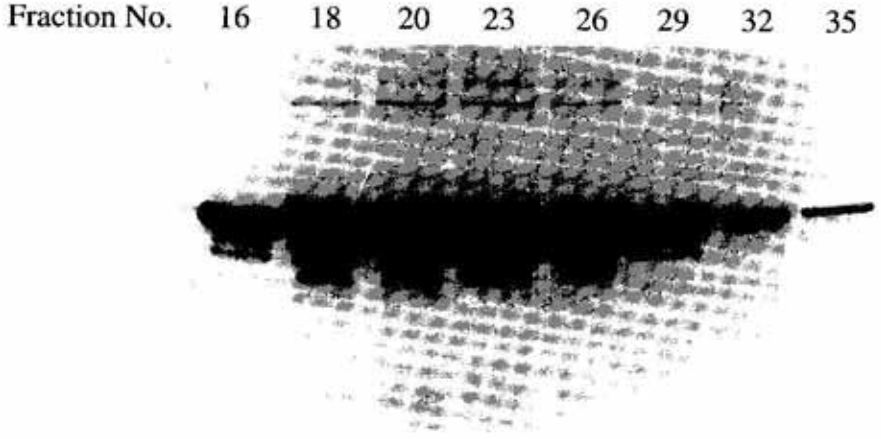
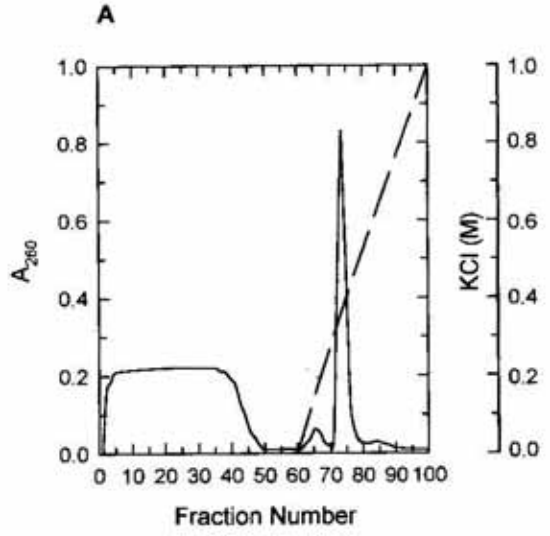


Figure 2. Purification of GST-Cys2 protein on agarose-glutathione.

The supernatant of crude extract was applied to the 3 mL agarose-glutathione column, and eluted with reduced glutathione as described under Materials and Methods. On individual lanes of the 12% polyacrylamide/SDS gel 1 μL of collected fractions (16–35) was loaded.

proteins are denatured or undergo conformational changes. Therefore we used UV spectroscopy to investigate the interaction of phospholipids and calcium with the Cys2 protein. The UV absorption spectra of Cys2 protein are shown in Fig. 5A. The absorbance of Cys2 protein at 205 nm was changed upon exposition to phosphatidylserine vesicles (100 nmol/mL) while addition of Ca^{2+} at concentration of 50 μM have no effect on UV spectra of Cys2 protein taken in the absence or in the presence of PS (Fig. 5A). Also when Cys2 protein was exposed to phosphatidylcholine or phosphatidylethanolamine vesicles no changes in UV spectra were observed. However, some lowering of absorbance at 205 nm was noted in the presence of phosphatidyl 4,5-bisphosphate (PIP_2) (Fig. 5B). Similar small changes in UV spectrum of Cys2 protein were also observed in the presence of phosphatidyl 4-phosphate but not in the presence of phosphatidylinositol (not shown). The effect of PIP_2 on UV spectra of Cys2 although small, depended on Ca^{2+}. Exposition of the Cys2 protein to PIP_2 in the presence Ca^{2+} resulted in a larger change of absorbance at 204 nm than in the absence of Ca^{2+} (Fig. 5B).

Addition of phorbol 12,13-dibutyrate (PDB) to aqueous solution of Cys2 in the absence of lipid micelles resulted in a rapid precipitation of the protein. Therefore, in order to investigate the interaction of PDB with Cys2 protein it was necessary to include 25% acetonitrile in the incubation solution to stabilize Cys2 protein as well as Cys2-PDB complex. The UV absorption spectra of Cys2 protein in 25% CH_3CN/75% H_2O resembled that taken in the absence of CH_3CN, but were shifted towards red by 5 nm and the intensity at 210 nm increased (Fig. 6A). A significant increase in absorbance of Cys2 protein observed in the presence of 25% CH_3CN could be the result of some relaxation of protein structure or deaggregation of Cys2 molecules. Characteristic property of lipid-dependent activation of PKC is its stereospecificity. For instance, PKC is activated specifically by 4β-hydroxy-PDB but not by its 4α-stereoisomer [26]. Previously, it was shown that these aspects of PKC activation are conserved within the Cys2 domain [8, 9]. Consistently the data presented in Fig. 6B indicate that only 4β-hydroxy 12,13-dibutyrate but not 4α-hydroxy 12,13-dibutyrate



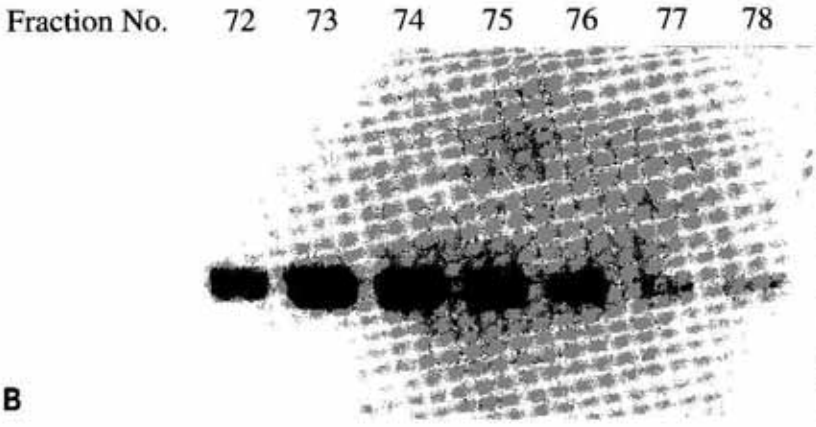


Figure 4. Purification of Cys2 protein on cation exchange FPLC column.

The dialyzed solution of Cys2 protein was applied to a Mono S column (HR 5/5). The column was washed and eluted as described under Materials and Methods. **A**, elution profile of Cys2 from Mono S column. **B**, 15% polyacrylamide/SDS gel of the fractions eluted between 310–325 mM KCl.

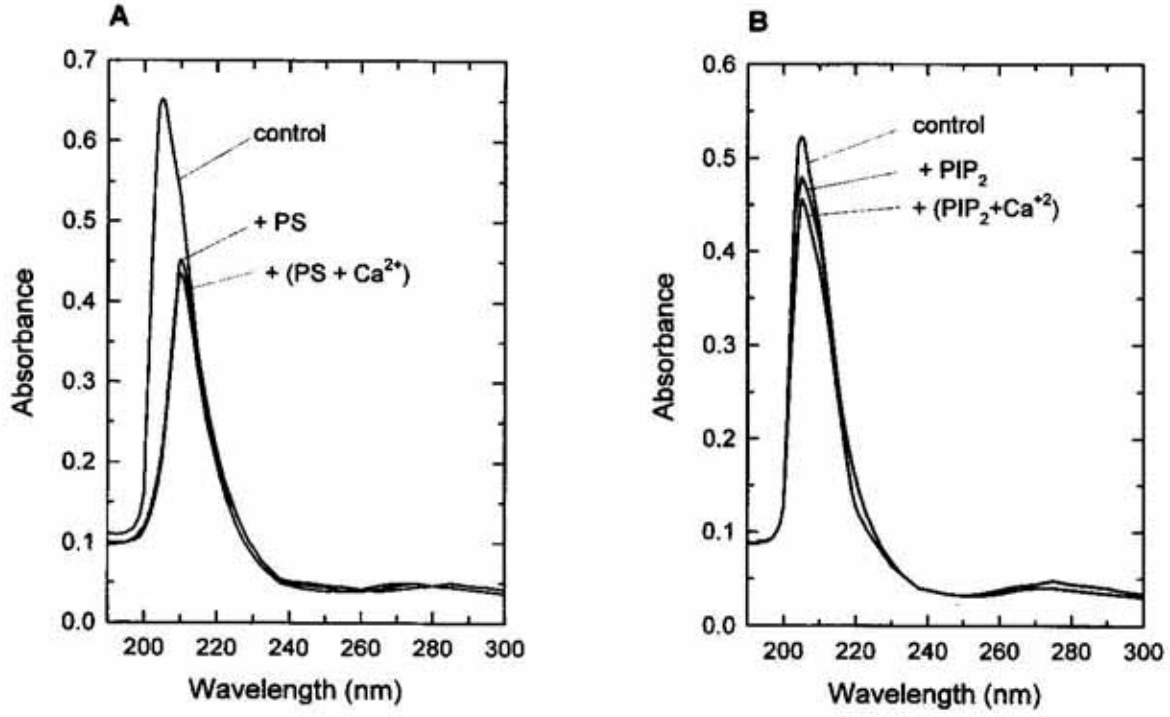


Figure 5. Effect of phospholipids and calcium ions on the UV spectra of Cys2 protein measured: A, 50 mM Hepes/NaOH, pH 7.4, 100 mM KCl, (control) with 100 nmol/mL phosphatidylserine vesicles, (PS); with 100 nmol/mL phosphatidylserine vesicles and 50 μM Ca^{2+}, (PS + Ca^{2+}); B, in control with 100 nmol/mL phosphatidylinositol 4,5-bisphosphate vesicles, (PIP_2); with 100 nmol/mL phosphatidylinositol 4,5-bisphosphate vesicles and 50 μM Ca^{2+}, (PIP_2 + Ca^{2+}).

The measurements were performed with about 60 μg (A) and about 50 μg (B) of Cys2 protein.

produced changes in UV absorption spectrum of Cys2 protein. Exposition of Cys2 protein to PDB changed absorbance at the 210 nm region (Fig. 6B). Including PS with the PDB had marked effect on UV spectrum of Cys2 protein (Fig. 6B). Conversely, the UV absorption spectra of Cys2 protein taken in the presence of PS and in the presence of PS + PDB were the same (Fig. 6B). The PIP_2 had no effect on UV spectra of Cys2 protein in 25% CH_3CN/75% H_2O (not shown).

Binding to phospholipid vesicles

In order to evaluate the functional significance of phospholipid interaction with Cys2 protein we examined binding of phospholipid vesicles with Cys2. It was previously reported that the lipid binding activity of the thrombin cleaved Cys2 protein is unstable [9]. For this reason we characterized the binding properties of the Cys2 protein with diacylglycerol (DAG) and phorbol ester. Data presented in Fig. 7 indicate that phorbol ester binds to Cys2 protein in a highly stereospecific manner. Stimulation of binding of Cys2 protein to PS+PC (5:5) vesicles was only observed for 4β-hydroxy 12,13-dibutyrate but not for 4α-hydroxy 12,13-dibutyrate. Similar results were obtained for stereoisomers of diacylglycerol. Only 1,2-dioctanoylglycerol but not 1,3-dioctanoylglycerol stimulated Cys2 binding to phospholipid vesicles (Fig. 8). Basing on these results we concluded that the Cys2 protein used in our experiments was fully functional with respect to lipid binding. In order to evaluate the importance of weak interactions of PIP_2 and PIP with Cys2 protein detected by UV study (Fig. 5B), we examined binding of Cys2 protein to phospholipid vesicles containing either PIP_2 or PIP. Data presented in Fig. 8 indicate that neither PIP_2 nor PIP

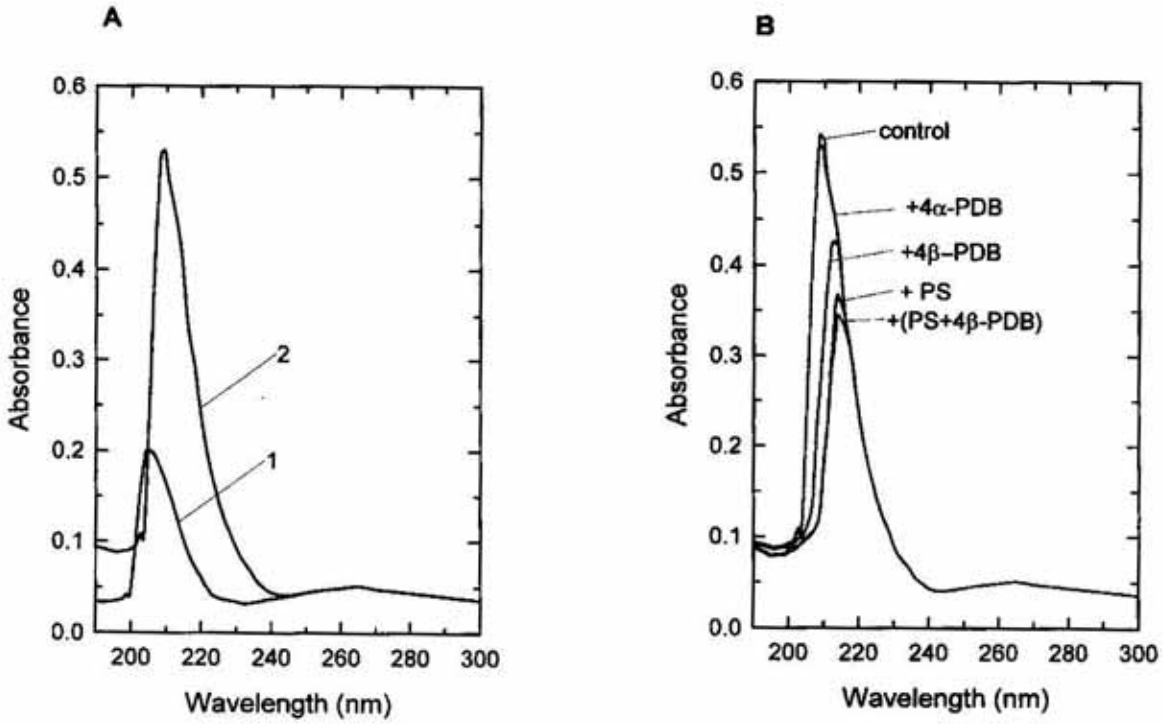


Figure 6. The UV spectra of Cys2 protein measured: A, in 50 mM Hepes/NaOH, pH 7.4, 100 mM KCl (1); with 25% CH_3CN added (2); B, in the buffer supplemented with CH_3CN (control) and additionally with, 4α-phorbol 12,13-dibutyrate (4α-PDB); 4β-phorbol 12,13-dibutyrate (4β-PDB); phosphatidylserine (PS), phosphatidylserine and 4β-phorbol 12,13-dibutyrate (PS + 4β-PDB).

The measurements were performed with about 20 μg of Cys2 protein.

stimulated binding of Cys2 protein to phospholipid vesicles.

DISCUSSION

The Cys2 protein used in our study was highly expressed due to the use of pGEX-2T vector and highly purified owing to the efficient cleavage of GST-Cys2 fusion protein and efficient chromatography. Previously reported methods of GST-Cys2 expression in bacterial system relied on the use of pGEX-2T vector [8, 9, 27]. Obtained fusion protein was poorly digestible by thrombin. Here, we used the modified vector, pGEX-2TKG with the incorporated glycine spacearm prior to the thrombin cleavage site [20]. This affords rapid and complete cleavage of the fusion protein with the only three unrelated amino acids remained at the N-terminus. The high yield of soluble protein depended crucially on the conditions used for protein expressions. A large increase in yield of soluble GST-Cys2 was obtained when the temperature of expression was lowered to 23°C. It was reported previously that lowering the temperature and increasing the time of expression in Prokaryota tends to increase the yield of active enzyme [28–30].

The activation of conventional protein kinase C isozymes is critically dependent on DAG, Ca^{2+} and PS. Besides DAG and PS, several negatively charged lipids such as PIP_2, PIP, PI or PA are also capable of partial PKC activation [14–18]. Studies with the use of second cysteine-rich region (Cys2) expressed in *E. coli* cells as a fusion protein with GST showed that Cys2 domain of PKC-γ contains regions necessary and sufficient for lipid-dependent interaction with phorbol esters and PS [8, 9, 27]. The results presented in this

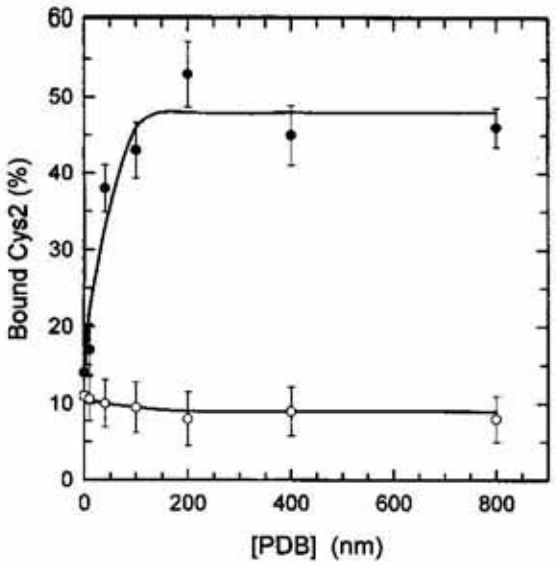


Figure 7. Binding of Cys2 protein to phospholipid vesicles (PC + PS) (5:5) in the the presence of 4α-phorbol 12,13-dibutyrate (○) and 4β-phorbol 12,13-dibutyrate (●).

Incubations were conducted at 22°C. Protein bound was assessed as described in Materials and Methods. The data show means ±S.D. (n = 4).

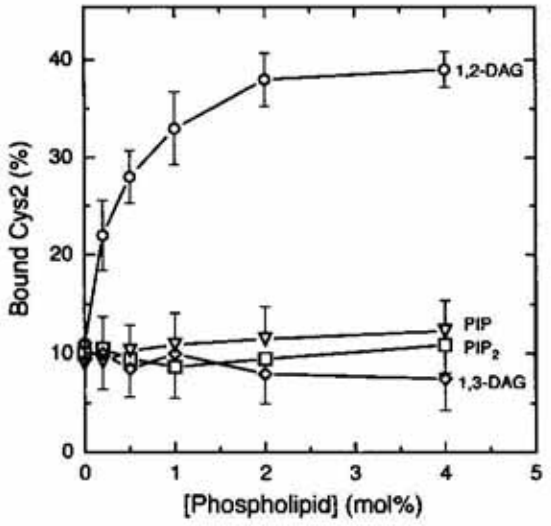


Figure 8. Binding of Cys2 protein to phospholipid vesicles (PC + PS) in the presence of: 1,2-dioctanoylglycerol (○, 1,2-DAG), 1,3-dioctanoylglycerol (◇, 1,3-DAG), phosphatidylinositol 4,5-bisphosphate (□, PIP_2), phosphatidylinositol 4-phosphate (△, PIP) at concentrations indicated.

Protein bound was assessed as described in Materials and Methods. The data show means ±S.D. (n = 3).

report indicate that UV absorption of Cys2 protein in the region of 200–210 nm is greatly affected by the presence of PS (Fig. 5). Conformational changes in proteins result in alterations of protein absorbance at 200–230 nm. Changes in protein structure that lead to burying chromophores in the non-polar environment decrease the UV absorption in this region of wavelength. Moreover, changes in protein structure should be also taken into consideration [31]. In general the protein UV absorption decreases with the assembly of α-helical conformation [32, 33]. The NMR studies of PKC-γ Cys2 domain showed that Cys2 has five β-strands and one α-helix (Scheme 1). There are two type I β-turns stabilized by two zinc atoms. The binding sites for phorbol are provided by the residues S111, T113, F114, L122, Y123 and G124. The lipids interact with amino acids involved in phorbol binding and L116, H117, S118, Y119, S120, S121 and the residues forming the binding site 1 for zinc (H102, C132) and site 2 (C115, C118, 138–145, 147) [12]. Observed by us large change in Cys2 absorbance in the 205 nm region in the presence of PS might be a consequence of conformational changes of protein. However, our previous study on interaction of Cys2 protein and PS with the use of NMR showed that binding of PS molecules to Cys2 do not induce any conformational changes of Cys2 [12]. On the other hand, in binding of Cys2 protein with the lipids two tyrosine, one phenylalanine and two histidine residues are involved. These facts taken together suggest that the change in absorbance at 205 nm observed in the presence of PS might be associated with the perturbation of the tyrosine, phenylalanine and histidine environment. It could be assumed that PS interacts with Cys2 domain mainly at the phorbol binding site. However, exposition of Cys2 protein to PDB resulted in smaller changes in UV absorption spectrum compared to those induced by PS (Fig. 6B). Moreover, the effect of PS and PDB on the absorption spectrum of Cys2 protein

were not additive. Exposition of Cys2 protein to PS or PS + PDB induced the same changes in UV spectrum of the protein (Fig. 6B). Similar results were observed with the use of heteronuclear multidimensional NMR [12]. In the work cited it was observed that including PDB with PS had little discernible effect on most of the spectrum. Thus, it might be concluded that PS comparing to PDB interacts with a larger area of Cys2 protein, and that the binding sites for these two molecules are at least overlapping. However, it should be kept in mind that due to insolubility of Cys2-PDB complex we had to use organic solvent, which may have altered the protein structure and reduced the binding affinity of PDB. The lack of Ca^{2+} effect on PS-induced changes in UV spectra of Cys2 (Fig. 5) indicate that the interaction of Cys2 domain with PS vesicles does not depend on Ca^{2+}.

No changes in UV spectra of Cys2 protein exposed to PE, PC or PI were observed. This may indicate that the reported previously [15, 16] regulatory effect of these phospholipids relay on the interaction with other domain of PKC-γ than of Cys2. The very small changes in UV absorption of Cys2 observed in the presence of PIP and PIP_2 indicated that the interaction of phosphatidylinositol molecules with Cys2 domain does not occur in the Cys2 region that binds PS and phorbol esters. On the other hand, the observed lack of PIP_2 and PIP ability to stimulate binding of Cys2 protein to phospholipid membranes indicate the weak interactions of PIP_2 and PIP with Cys2 protein detected by UV spectroscopy are not functionally significant. Thus, we conclude that PIP and PIP_2 activates PKC-γ by mechanisms different from that of DAG/phorbol ester, and that the site of PIP and PIP_2 interaction with PKC-γ is distinct from that of the phorbol ester binding site. Also it is possible that PIP_2, PIP and PI activate PKC-γ mainly by binding to other domain of the kinase.

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