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Structure of human protein kinase C eta (PKCη) C2 domain and identification of phosphorylation sites

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Abstract

Protein kinase C eta (PKCη) is one of several PKC isoforms found in humans. It is a novel PKC isoform in that it is activated by diacylglycerol and anionic phospholipids but not calcium. The crystal structure of the PKCη-C2 domain, which is thought to mediate anionic phospholipid sensing in the protein, was determined at 1.75 Å resolution. The structure is similar to that of the PKC epsilon C2 domain but with significant variations at the putative lipid-binding site. Two serine residues within PKC eta were identified *in vitro* as potential autophosphorylation sites. In the unphosphorylated structure both serines line the putative lipid-binding site and may therefore play a role in the lipid-regulation of the kinase.

Keywords: Protein kinase C; PKC; Eta; C2 domain; Structure; Phosphorylation; Novel isoform; X-ray

The human protein kinase C (PKC) family is grouped into classical (α, βI, βII, and γ), novel (δ, ε, η, and θ) and atypical PKC isoforms (ξ, and ι/λ). Usually existing as inactive cytosolic forms, PKCs become activated upon translocation to membranes in the presence of isoform-specific second messengers [1,2]. Classical PKCs, for example, are primed by heterologous upstream growth factor related kinases and activated by the presence of diacylglycerol (DAG) and calcium ions, which are common downstream signals of G protein coupled receptors. These second messengers help target the kinase to plasma membranes or isoform-specific intracellular membranes [3], where substrates reside. Exogenous tumor promoting phorbol esters can also activate PKCs by eliminating the requirement for DAG [2]. In contrast to classical isoforms, novel PKCs are independent of calcium regulation [4] and atypical PKCs are independent of both calcium and DAG [1,2]. Cofactor dependences are thought to be largely mediated through the N-terminal regulatory domains of the protein.

The mechanism of kinase activation is coupled with membrane targeting. In the inactive cytosolic form an inhibitory pseudosubstrate peptide, located near the N-terminal regulatory domains, suppresses the activity of the kinase domain through its direct interaction with the active site. The classic and novel PKCs have two types of regulatory domains: the C1 and C2 domains [5]: the C1 domain binds DAG and phorbol esters, and the C2 domain binds phospholipids head groups in a calcium dependent (classical isoforms) or independent (novel isoforms) manner. Binding of C1 and C2 domains to membranes is coupled with release of pseudosubstrate, but the exact mechanism of this transition remains unclear [6]. A single domain-membrane interaction may be sufficient for targeting, but release of pseudosubstrate-mediated inhibition appears to require both domains to bind [7]. The amount and duration of these second messengers therefore plays an important role in PKC activation [7]. Although a

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structure of a classical C2–calcium–phospholipid complex is available [8], the mechanism by which novel C2s interact directly with phospholipids remains unknown.

PKCη is a novel PKC isoform highly enriched in epithelial tissues such as skin and lung [9,10]. Multiple lines of evidence suggest that PKCη may be involved in regulating the proliferation and remodeling of epithelial cells [11]. Mice in which this protein is knocked out show a delayed wound healing response and an increase in skin tumor formation on exposure to carcinogens [11]. While in keratinocytes PKCη may be involved in both differentiation [12] and cell growth [13].

Here, we present the structure of the regulatory C2 domain from human PKCη. This domain is thought not to effect substrate-specificity of the kinase [14] but instead aid in its localization to membranous systems. The equivalent domain from rat PKCε has previously been solved [15]. Differences are observed in the loop regions C-terminal to the two α-helices within the structures. Through treatment with the kinase domain it was shown *in vitro* that PKCη contains two autophosphorylation sites within its C2 domain. These were identified by mass spectrometry as residues Ser-28 and Ser-32, both of which reside in helix α1. This helix is near the putative lipid-binding site of the domain [15], consequently autophosphorylation at these residues may act as a means of self-regulation in PKCη. Ser-28, but not Ser-32, is conserved in PKCε so autophosphorylation may be a means of regulating the function of the C2 domains of multiple PKC family members.

Materials and methods

Cloning and protein purification. Using the mammalian gene collection (MGC) clone with accession code BC037268 as the cDNA template for the human PKCη isoform, two constructs (1–138 and 350–682) were amplified by PCR and inserted into the p28-LIC expression vector using the InFusion ligation independent cloning system (BD biosciences). The resulting vectors were transformed into *Escherichia coli* BL21 (DE3) cells (Stratagene). Expression from these plasmids yields the N-terminal fusion of the 20 amino acids MGSSHHHHHHSSGLVPRLGS containing a hexahistidine tag (his-tag) and thrombin cleavage site to each construct. Two liters of Terrific Broth media containing 100 μg/mL Kanamycin was inoculated and aerated using the LEX system (http://sgc.utoronto.ca/Technology/lex.php) at 37 °C to OD600 ~ 4 before induction at 15 °C using 50 μg/mL IPTG for 14–15 h. At 4 °C the cell-pellet was resuspended in 50 mL of binding buffer (50 mM Tris–HCl, 0.5 M NaCl, and 0.5 mM TCEP, pH 8.0) containing Sigma's protease inhibitor cocktail (P2714-1BTL), homogenized for 30–60 s on ice, and lysed by sonication (Virtis408912, Virsonic) on ice. The resulting lysate was clarified by centrifugation and applied to 4 mL Ni–NTA agarose affinity resin (Qiagen #30250). The Ni–NTA resin was washed with 50 mL of binding buffer and 50 mL of wash buffer (50 mM Tris–HCl, 500 mM NaCl, 20 mM imidazole, and 0.5 mM TCEP, pH 8.0) before eluting into 7 mL of elution buffer (50 mM Tris–HCl, 500 mM NaCl, 250 mM imidazole, and 0.5 mM TCEP, pH 8.0). The his-tag was removed from the C2 domain prior to gel-filtration through incubation for 1 h at room temperature with 6 U human plasma thrombin (Sigma #T7009) per mL of eluate. Using an AKTAxpress (GE Healthcare) the eluate was loaded onto an XK 16 × 65 column packed with HighLoad Superdex 200 (Pharmacia #17-1043-02) resin pre-equilibrated in binding buffer. The PKCη-C2 domain eluted as a single major peak, the fractions from this peak were pooled and concentrated to 1.4 mg/mL using an Amicon YM10 centrifugal filter device. The PKCη-KD eluted as a broad peak, fractions were collected and concentrated to ~10 mg/mL. Both constructs were frozen in liquid nitrogen and stored at −80 °C until required.

Crystallization and structure of the C2 domain. A 3 μL volume of protein at 1.1 mg/mL was mixed with 3 μL of reservoir solution that consisted of 22% w/v polyethylene glycol 3350 and 0.2 M $CaCl_2$. The drop was sealed over 1 mL of reservoir and allowed to reach equilibrium at 18 °C, and crystals grew overnight. The crystal was soaked for 1–2 min in a cryo-protecting solution consisting of reservoir plus 300 mg/mL D-glucose then frozen in liquid nitrogen and mounted at 100 K for X-ray diffraction studies. Diffraction data were obtained from these crystals to 1.75 Å resolution using our home source FR-E generator and RAXIS IV++ detector. A molecular replacement solution using the PKCε-C2 domain as a molecular replacement probe (PDB code 1GMI) [15] was found using the program Phaser [16]. Automatic model building with ARP/wARP [17], TLS [18], and refinement using REFMAC5 [17], as well as manual building with the graphics program O [19], led to a model with a working R value of 0.18 and a free R value of 0.22 for the resolution range from 1.75 to 19.9 Å. The final model consists of protein residues 5–137 and seven extraneous residues from the N-terminal his-tag tag as well as 132 water molecules. The model has been deposited with the PDB code 2FK9.

Protein phosphorylation. A 1.5 mg/mL solution of the PKCη-C2 domain in gel-filtration buffer and with the his-tag removed was brought to a final concentration of 5 mM ATP and 10 mM $MgCl_2$. A 10 mg/mL stock solution of recombinant his-tagged PKCη-KD was then added giving a final concentration 0.01 mg/mL (approximately a 1:30 molar ratio). The sample with PKCη-KD added and a control without PKCη-KD addition was incubated at room temperature, and 5 μL aliquots were removed and analyzed by ES-TOF/MS at 2, 4, 6, 8, 24, and 30 h incubation time. The reaction mixture was stopped after ES-TOF/MS analysis detected no unphosphorylated PKCη-C2, usually at the 24 or 30 h time points depending on the activity of the PKCη-KD prep. The reaction was filtered through a 0.22 μm syringe-filter, passed over 1 mL Ni–NTA resin equilibrated in gel-filtration buffer, and reloaded on the gel-filtration column. Samples from the single elution peak were collected and concentrated to 0.6–1.0 mg/mL.

Enzymatic digestion and mass spectrometry. In solution digestions were performed on 5 μg of the purified protein using either 0.05 μg of trypsin (CalBiochem Inc.) or endoprotease Lys-C (Roche Diagnostics GmbH) in 25 mM ammonium bicarbonate, and each mixture of a total 10 μl solution was incubated at 37 °C for 12 or 2 h, respectively. The reaction was terminated by freezing the sample at −80 °C for subsequent MALDI MS analysis. Mass measurements of intact proteins were carried out on an Agilent 1100 LC/MSD TOF equipped with an ESI source operating in positive ionization mode. Following digestion by trypsin or Lys-C, the proteolytic products were examined on Applied Biosystems/MDS Sciex QStar XL QqTOF instrument equipped with an oMALDI™ two source. For mass determination by ESI, the protein sample was prepared in 50% methanol/water with 0.1% formic acid. Whereas in the case of MALDI MS analysis of the peptide digest, 2,5-dihydroxybenzoic acid (150 mg/ml) dissolved in acetonitrile/water (v/v, 1:1) was used as the matrix and the sample was deposited on a stainless steel MALDI target. The MALDI MS data were acquired using a nitrogen laser (337 nm) operated at 20 Hz, and the power was set to 17 μJ. Mass scale m/z was calibrated externally with two standard peptides (dalargin and melittin). Argon was used as the collision gas in the MS/MS experiments, and collision energies were manually adjusted to generate optimum fragmentation of the parent ions. MALDI MS and MS/MS spectra were acquired and processed using Applied Biosystems/MDS Sciex Analyst QS and BioAnalyst™ Software 1.1. The masses of peptides derived from *in silico* trypsin cleavage of the PKCη-C2 domain sequence were calculated using Protein Prospector (http://prospector.ucsf.edu) [20]. Masses were calculated allowing for 0, 1, and 2 missed endoprotease cleavage sites as well as phosphorylation of serine, threonine, and tyrosine residues.