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Initial three-dimensional reconstructions of protein kinase C δ from two-dimensional crystals on lipid monolayers ☆

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Abstract

Two-dimensional crystals of protein kinase C δ (PKCδ) and of its regulatory domain (RDδ) were grown on lipid monolayers and analyzed by electron microscopy at tilt angles varying from −50° to +55°. Although the crystals exhibit pseudo-3-fold symmetry, analysis of difference phase residuals indicates that there is only one way to align the crystals for merging so the data were processed in plane group P1. Three-dimensional reconstructions generated for several two-dimensional crystals each of PKCδ and RDδ show good agreement and are consistent with membrane attachment *via* a single C1 subdomain, a small surface contact by one or two loops from the C2 domain, and, in intact PKCδ, a small appendage from the catalytic domain, probably V5. Two-dimensional crystallography with three-dimensional reconstruction should be suitable for examination of additional PKC isozymes and for analysis of the enzymes bound to substrates and other proteins.

Keywords: PKC structure; Protein–lipid binding; C1 domain; C2 domain

1. Introduction

Protein kinase C (PKC)[1] family members are critical regulators of many cellular functions, from activity of specific receptors and ion channels to control of cell growth, development and apoptosis reviewed in 1–3]. Although some differences in substrate specificity of the isozymes have been determined based on interaction with a degenerate peptide library [4], there is significant overlap in substrate recognition sequences *in vitro*. In some cases, altering lipid composition can convert a poor substrate into a good one for a given pure isozyme [*e.g.*, 5]. It is becoming increasingly apparent that the subcellular localization of individual PKC isozymes controls their access to specific substrates. The subcellular localization is determined largely by the different N-terminal regulatory domains (RD) variably containing C1 and C2 subdomains that interact with membrane lipids and/or other proteins [reviewed in 6–8].

The C-terminal catalytic domain consists of ATP-binding C3 and protein substrate-binding C4 subdomains. The RD confers dependence on acidic phospholipids, classically phosphatidylserine (PS). In the RD of classical (c)PKCs (α, βI, βII, and γ), a pseudosubstrate sequence that occupies the active site of inactive enzymes is followed by two C1 subdomains, C1A and C1B, which bind diacylglycerols (DAG)s and/or phorbol ester tumor promoters, and a Ca^{2+}-binding C2 domain. Novel (n)PKCs (δ, ε, η, and θ) lack the Ca^{2+} requirement and have a variant C2 domain lacking Asp residues necessary for Ca^{2+} binding. This is followed by the pseudosubstrate and then C1A and C1B domains. The atypical (a)PKCs (ζ and λ/ι) lack both Ca^{2+} and diacylglycerol requirements and have only one C1 domain that does not bind phorbol esters. Short variable sequences (V1–V5) unique to each isozyme connect these

Abbreviations: PKC, protein kinase C; RD, regulatory domain; 3D, three-dimensional; 2D, two-dimensional; NMR, nuclear magnetic resonance; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; DO, diolein; DAG, diacylglycerol; FRC, Fourier ring correlation; DPR, difference phase residuals; PDB, protein data bank.

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constant domains. Association of PKCs with their activating lipids and/or binding proteins stabilizes an active conformation with the pseudosubstrate removed from the active site.

Individual PKC isozymes can have opposing effects in the same tissue — *e.g.*, PKCε is implicated in ischemic preconditioning of cardiac tissue whereas PKCδ is implicated in reperfusion injury [9]. PKCδ is involved in many apoptotic pathways and may function as a tumor suppressor in some situations [*e.g.* 10, reviewed in 11]. It has been implicated in oxidant-induced loss of intestinal epithelial barrier function [12] and its loss has been implicated in human squamous cell carcinomas [13]. Except for a relatively specific ATP-site PKCβ inhibitor (ruboxistaurin) that is effective in diabetic retinopathy and neuropathy [14, reviewed in 15], few clinically useful isozyme-specific inhibitors or activators are available; however, their development is an area of active pursuit [reviewed in 16–18]. Detailed analysis of the structure and activation of individual isozymes should facilitate design of more specific activators and inhibitors.

The structure of the catalytic domain of PKCβ was modeled [19] based on the similar structure of cyclic AMP-dependent kinase catalytic subunit [20] and more recently, structures of the catalytic domains of PKCs θ [21], ι [22], and β [23] have been determined by X-ray crystallography. Nuclear magnetic resonance (NMR) structures of the C2 domains of PKCβ and synaptotagmin were reported early on [24]. Structures have been determined by X-ray crystallography for the C2 domains of PKCs δ [25], β [26], α [27], ε [28] and η [29] and for the C1B domain of PKCδ [30]. Despite more than a decade of effort, adequate 3-dimensional (3D) crystals of intact PKCs have not yet been obtained, so the orientation of the C1A, C1B and C2 domains relative to each other, to the membrane, and to the catalytic domains has not been established. Crystals of intact PKCs obtained from solution would be expected to reveal the enzymes in their inactive pseudosubstrate-inhibited forms.

Previously we reported growth of two-dimensional (2D) crystals of several PKCs on lipid monolayers [31,32]. Although the crystals exhibit pseudo-three-fold symmetry, analysis of their computed and observed diffraction patterns precluded P3 symmetry and the unit cell dimensions are too small to contain more than one PKC molecule. The absence of any symmetry constraints with crystals in plane group P1 has made analysis much more difficult. We have used several approaches to circumvent the problems and now report initial 3D reconstructions of PKCδ and RDδ from electron microscopic analysis of 2D crystals on activating lipid monolayers.

2. Materials and methods

2.1. PKC expression and purification

PKCδ was expressed from baculovirus constructs in Sf9 insect cells and purified from cytosolic extracts through MonoQ and phenylsuperose high performance liquid chromatography columns basically as previously described [8,31–33]. Activity was measured by transfer of phosphate from ^{32}P-ATP to myelin basic protein as described previously [34]. Purification was assessed by electrophoresis through 10% polyacrylamide gels followed by silver staining and Western blotting with polyclonal antisera (Santa Cruz Biotechnology) directed at a sequence in the C-terminal V5 region of PKCδ as shown in Fig. 1.

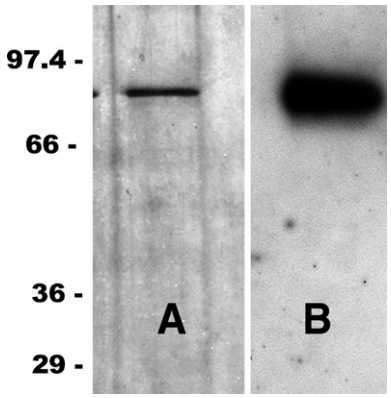

Fig. 1. Purity of PKCδ preparation. PKCδ expressed in Sf9 cells from baculovirus constructs and purified as described in the Materials and methods was electrophoresed through 10% polyacrylamide gels with 3% stacking gels and stained with silver nitrate (A) or blotted onto nitrocellulose and probed with PKCδ-specific antibody (B) and detected with enhanced chemiluminescence reagents. Lanes contained 29 ng protein. Molecular weight markers are indicated at the left.

RDδ, identified on the basis of its gel mobility and phorbol ester binding capacity, was obtained from a preparation that had undergone proteolysis. Both PKCδ and RDδ were quantified by their ability to bind ^{3}H-phorbol 12,13-dibutyrate (NEN Life Science products, Boston, MA) as described previously [35]. Preparations were stored in 30% glycerol at −70 °C. Aliquots were thawed immediately prior to addition to crystallization wells.

2.2. Specimen preparation and electron microscopy

2D crystals of PKCδ and RDδ were grown on lipid monolayers composed of dioleoylphosphatidylcholine (DOPC): dioleoylphosphatidylserine (DOPS): diolein (DO) [(45:50:5, molar ratio); all lipids from Avanti Polar Lipids, Alabaster, AL] as described previously [32]. Briefly, PKCδ (48 nM) or RDδ (5.4 nM) in 17 μl of 20 mM 3-(*N*-morpholino)-propanesulfonic acid, pH 7.8 was overlayed with 12 μl of the same buffer and then 1.0 μl of freshly mixed lipids (2.0 mg/ml in chloroform:hexane, 1:1) and incubated for 7 h in darkness at 4 °C. Membranes were picked up on carbon-coated lacey 300 mesh copper electron microscopy grids (Electron Microscopy Sciences) and stained with 1% Nanovan (Nanoprobes, Inc.) for 30 seconds, washed with distilled water, and dried for 1 h.

Images were taken with a TECNAI-12 electron microscope at an acceleration voltage of 100 keV with magnification of 67,000×, and at tilt angles of −50° to +55°. Real time Fast Fourier Transforms were applied for focusing and correction of astigmatism. An *oriented gold single crystal standard* (Electron Microscopy Sciences) was used to calibrate the microscope magnification.

2.3. Image analysis

Images were digitized at a resolution of 2.4 Å/pixel and the data were processed using the CALIDRIS, Inc. software package that includes three programs: Crystallographic Image Processing (CRISP) [36]; TriMerge for merging of tilted images; and eMap for visualization of 3D reconstructions. All processing results were checked with MRC-IMAGE, ICE, and SPIDER/Web packages [37]. Selection of raw field images was based on an estimation of the quality of the Fourier power spectra. Lattice distortion correction and contrast transfer function determinations of crystalline areas were performed for the untilted images [38].

Although (0,1), (1,0) and (−1,1) reflections had similar absolute phase values, one had lower intensity inconsistent with P3 symmetry. Analysis by calculation of Fourier ring correlations (FRC) and difference phase residuals (DPR) performed with the SPIDER/Web package [37] showed that only one of six possible orientations of two crystals with respect to each other was appropriate for merging; thus processing was carried out in P1. Since the phase