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Individual C1 domains of PKD3 in phorbol ester-induced plasma membrane translocation of PKD3 in intact cells

Gülsüm Anderson, Jun Chen, Q. Jane Wang*

Department of Pharmacology, University of Pittsburgh School of Medicine, E1354 Biomedical Science Tower, Pittsburgh, PA 15261, USA

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Abstract

Protein kinase D3 is a novel member of the serine/threonine kinase family PKD. The regulatory region of PKD contains a tandem repeat of C1 domains designated C1a and C1b that bind diacylglycerol and phorbol esters, and are important membrane targeting modules. Here, we investigate the activities of individual C1 domains of PKD3 and their roles in phorbol ester-induced plasma membrane translocation of PKD3. Truncated C1a of PKD3 binds [^{3}H]phorbol 12, 13-dibutyrate with high affinity, but no binding activity is detected for C1b. Meanwhile, mutations in C1a of truncated C1ab of PKD3 lead to the loss of binding affinity, while these mutations in C1b have little impact, indicating that C1a is responsible for most of the phorbol ester-binding activities of PKD3. C1a and C1b of the GFP-tagged full length PKD3 are then mutated to assess their roles in phorbol ester-induced plasma membrane translocation in intact cells. At low concentration of phorbol 12-myristate 13-acetate (PMA), the plasma membrane translocations of the C1a and C1ab mutants are significantly impaired, reflecting an important role of C1a in this process. However, at higher PMA concentrations, all C1 mutants exhibit increased rates of translocation as compared to that of wild-type PKD3, which parallel their enhanced activation by PMA, implying that PKD3 kinase activity affects membrane targeting. In line with this, a constitutive active PKD3-GFP translocates similarly as wild-type PKD3, while a kinase-inactive PKD3 shows little translocation up to 2 μM PMA. In addition, RO 31-8220, a potent PKC inhibitor that blocks PMA-induced PKD3 activation in vivo, significantly attenuates the plasma membrane translocation of wild-type PKD3 at different doses of PMA. Taken together, our results indicate that both C1a and the kinase activity of PKD3 are necessary for the phorbol ester-induced plasma membrane translocation of PKD3. PKC, by directly activating PKD3, regulates its plasma membrane localization in intact cells.

Keywords: C1 domain; PKD3; PKC; Phorbol ester; Membrane translocation

1. Introduction

Protein kinase D (PKD) is a novel serine/threonine kinase family that is now classified as a member of the Ca^{2+}-Calmodulin kinase superfamily [1]. It consists of PKD/PKCμ [2,3] and two newly identified kinases, PKD2 [4] and PKD3 (PKCν) [5]. PKD has been implicated in regulating a variety of cellular functions including Golgi function and organization, protein secretion, metastasis, apoptosis, and cell migration [6,7]. It is primarily localized in cytosol, some in the nucleus, Golgi and mitochondria [8], and is activated in intact cells by a range of stimuli including the tumor-promoting phorbol esters, G-protein coupled receptor (GPCR) agonists, and growth factors [6,7]. Meanwhile, PKD in intact cells can be activated via PKC-dependent mechanisms. PKC was found to directly bind, phosphorylate and activate PKD, and novel PKCs were implicated in the process [9,10]. PKC-mediated PKD1 activation involves the phosphorylation of two specific serine residues, Ser^{744} and Ser^{748}, in the activation loop of PKD1 catalytic domain [9–11]. The regulatory domain of PKD is comprised of several conserved structural motifs, in particular a C1 domain and a pleckstrin homology domain (PH). It has been implicated that both C1 and PH domains are important in regulating PKD1 kinase activity since deleting either domain leads to PKD1 kinase activation [12,13]. Accordingly, the phosphorylation of the activation

* Corresponding author. Tel.: +1 412 383 7754; fax: +1 412 648 1945.
E-mail address: qjw1@pitt.edu (Q.J. Wang).

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loop by PKCε serves to relieve the autoinhibition by the PH domain [11].

The C1 domain of PKD, similar to that in PKC, consists of a tandem repeat of cysteine-rich, zinc finger-like motifs designated as C1a and C1b that are responsible for the binding of the endogenous *sn*-diacylglycerol (DAG) and the phorbol ester tumor promoters [14,15]. It possesses a conserved 50 amino acid structural motif HX12-CX2CX13/14CX2CX4HX2CX7C (C, cysteine; H, histidine; X, any other amino acid) that coordinates two Zn^{2+} ions [15]. The 2 histidines and all but 1 of the 6 conserved cysteines coordinate the two Zn^{2+} ions. A number of residues such as those at the 3, 8, 11, 21, 24, 27, and 38 positions have been shown to be critical for maintaining the overall structure and ligand binding by the C1 domain [14–16]. The C1 domain of PKD1 binds DAG and phorbol esters. However, the phorbol ester-binding activities of individual C1a and C1b domains of PKD1 are controversial. Dissimilar phorbol ester-binding capabilities of C1a and C1b of PKD1 have been described with the C1b domain responsible for most of the phorbol ester-binding activities in vivo and in vitro [17], which is in contrast to the report that C1a and C1b of PKD1 bound equally well to phorbol esters [18].

The C1 domain, by binding to DAG/phorbol esters, serves as an important membrane-targeting module for a variety of proteins in intact cells. For PKC, this domain plays a predominant role for the plasma membrane targeting of classical and novel PKC isoforms [19]. For PKD, however, membrane targeting appears to be regulated by multiple mechanisms. Based on the studies on PKD1, it has been shown that the C1 domain is responsible for localizing PKD1 to the plasma membrane in response to GPCR agonists, BCR ligation, and phorbol esters [20]. However, it is unclear in terms of the relative contributions of C1a and C1b of PKD1 in the process. Besides C1 domains, other mechanisms have also been reported to contribute to the membrane association of PKD1. These include mechanisms that regulate the PKD kinase activity and its interactions with other protein-binding partners [20–22]. PKC, the kinase that directly activates PKD in cells, was found to modulate the duration of plasma membrane localization of PKD1. Rey et al. revealed that although PKC activity was not required for the plasma membrane association of GFP-PKD in response to bombesin-induced GPCR activation, it was necessary for the dissociation of PKD1 from the plasma membrane in Swiss 3T3 and MDCK cells [20]. This reverse translocation from the plasma membrane to the cytosol was later found to require the phosphorylation of the activation loop and subsequent activation of PKD1 by PKC [20,21]. Meanwhile, protein-binding partners of PKD1 have also been reported to modulate its membrane association. Oancea et al. demonstrated that the activation of the Gαq-coupled receptor induced persistent membrane association and activation of PKD1 [22]. Although the initial translocation and activation step requires the binding of diacylglycerol to the C1 domain, PKD1, once activated, was latched in its active state at the plasma membrane through direct binding to Gαq [22]. Compared to the well-characterized PKD1, the dynamics of intracellular targeting of PKD2 and PKD3 are largely undefined.

In this study, we have investigated the mechanisms that contribute to the phorbol ester-induced plasma membrane translocation of a novel PKD isoform, PKD3. We evaluate first that phorbol ester binding activities of individual C1a and C1b domains of PKD3 and their contributions to the phorbol ester-induced plasma membrane translocation of PKD3. C1a but not C1b of PKD3 binds PDBu with high affinity, indicating that C1a is responsible for the majority of the phorbol ester-binding activities of PKD3. Our time-lapse fluorescent live cell imaging analysis on mutants of the individual C1 domains reveals dissimilar roles of C1a and C1b in targeting PKD3 to the plasma membrane. In addition to the C1 domain, membrane targeting of PKD3 requires its kinase activity, which is regulated by PKC in intact cells.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA) and Phorbol 12, 13-dibutyrate (PDBu) were obtained from LC laboratory (Woburn, MA). [20-^{3}H]Phorbol 12, 13-dibutyrate ([^{3}H]PDBu) (20 Ci/mmol) was purchased from MP Biomedicals, Inc. (Irvine, CA). L-α-phosphatidylserine was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Reagents for expression and purification of glutathione *S*-transferase (GST) fusion proteins were purchased from Amersham Biosciences Corp. (Piscataway, NJ). Lipofectamine PLUS, cell culture medium and reagents were all purchased from Invitrogen (Gaithersburg, MD).

2.2. Cloning and mutagenesis of PKD3

The PKD3 cDNA cloned in the pGEM-Teasy vector was described previously [5]. For mammalian expression, the full length PKD3 cDNA was subsequently cloned directly into the XhoI-NotI sites of a pcDNA3.1/myc-His B vector. To generate the green fluorescent protein (GFP) fusion construct, PKD3 was amplified by PCR and subcloned into a XhoI-MluI site of a pEGFP-N1 vector (Clontech, Palo Alto, CA), modified by inserting a MluI linker into the plasmid digested with SmaI.

The point mutations in PKD3 were prepared using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The C1a mutant of PKD3, PKD3-C1a(P165G)-GFP, was generated by mutating proline at 165 position to glycine (P165G) in C1a domain. The C1b mutant, PKD3-C1b(P282G)-GFP, was generated by mutating proline at 282