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Scanning Mutagenesis Studies Reveal Multiple Distinct Regions within the Human Protein Kinase C Alpha Regulatory Domain Important for Phorbol Ester-dependent Activation of the Enzyme

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While phorbol ester-binding sites within protein kinase C alpha (PKCα) have been identified and characterized utilizing fragments of the enzyme, it remains unclear whether additional regions within the enzyme may play an important role in its ability to be activated by phorbol ester. To examine this hypothesis, we generated 20 glutathione-*S*-transferase-tagged, V1-deficient, human PKCα holoenzyme constructs in which tandem six or 12 amino acid residue stretches along the full regulatory domain were changed to alanine residues. Each protein was assessed for its ability to bind phorbol ester and to induce growth repression when its catalytic activity was activated by phorbol ester upon expression in yeast cells. Mutagenesis of residues 99–158 potently reduced phorbol binding, consistent with previously published findings on the importance of the C1b region in phorbol binding. In addition, we identified a number of regions within the PKC regulatory domain that, when mutagenized, blocked the activation of PKC-mediated growth repression by phorbol ester while actually enhancing phorbol ester binding *in vitro* (residues 33–62, and 75–86). This study thus helps distinguish regions important for phorbol binding from regions important for the ability of phorbol ester to activate the enzyme. Our findings also suggest that multiple regions within C2 are necessary for full activation of the enzyme by phorbol ester, in particular residues 231–254. Finally, three regions, when mutagenized, completely, blocked catalytic domain activity *in vivo* (residues 33–62, 75–86, and 123–146), underscoring the important role of regulatory domain sequences in influencing catalytic domain function, even in the absence of the V1 region containing the pseudosubstrate sequence. This is the first tandem mutagenesis study for PKC that assesses the importance of regions for both phorbol binding and for phorbol-dependent activation in the context of the entire holoenzyme.

Keywords: protein kinase C; phorbol ester; phorbol binding; PKC activation; regulatory domain

Introduction

Protein kinase C (PKC) is a family of phospholipid-dependent, Ser/Thr protein kinases, whose isoforms can be activated by diacylglycerol (DAG), or calcium ions.[1–3] The structures and functions of PKC isoforms have been studied and reviewed extensively, with each isoform consisting of conserved (C1–C4) and variable (V1–V5) regions.[1,4–9] PKC activity is thought to be self-inhibited by interaction of the highly cationic pseudosubstrate sequence within region V1 with the substrate-binding cleft of the catalytic domain, thereby blocking substrate access and catalytic activity (the V1 clamp model).[10–14] Binding of DAG and Ca^{2+} to the C1 and C2 regions of the regulatory domain, respectively, are thought to alter PKC conformation, releasing the pseudosubstrate sequence from the

Abbreviations used: DAG, diacylglycerol; PKC, protein kinase C; PDBu, phorbol dibutyrate; PMA, phorbol 12-myristate-13-acetate; GST, glutathione-*S*-transferase.

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catalytic cleft and allowing substrate access. The C1 region is composed of two tandem repeats of cysteine-rich zinc-finger domains (C1a and C1b), both of which have the capacity to bind DAG when expressed as individual protein fragments.[15–17] Phorbol esters such as phorbol 12-myristate-13-acetate (PMA) mimic the structure of DAG and can bind to proteins comprised of either the C1a or C1b domains. The C2 region binds both Ca^{2+} and phosphatidylserine, and plays a role in binding of the enzyme to phosphatidylserine-abundant membranes.[18–21] Although only the C2 domain has phosphatidylserine-binding sites,[22] both the C1 and C2 domains are believed to be involved in membrane translocation and subsequent enzyme activation.[23]

The C1a and C1b domains each span approximately 50 amino acid residues and consist of six conserved cysteine residues and two conserved histidine residues.[24,19,25,26] Controversy exists regarding the binding capacity of the C1a and C1b domains for DAG and phorbol esters. A number of previous studies have shown that recombinant proteins representing the C1a and C1b regions of PKC bind both phorbol esters and DAG with essentially the same affinity as that of the PKCα holoenzyme.[17,19,26,27] In contrast, other studies suggest strongly that the C1a region of PKCα possesses a higher affinity for binding DAG, while the C1b region binds phorbol ester preferentially.[20,28] In another study, phorbol ester binding assays performed with various lengths of C1a or C1b peptides showed differential binding to either the C1a or C1b peptide, depending on conditions.[29]

The identification and characterization of ligand-binding sites within PKC have been critical in defining structure–function relationships. The precise residues required for phorbol ester binding to the C1 region of PKC *in vitro* have been identified using deletion mutagenesis experiments,[19,26,30–32] or point mutagenesis.[19,33–35] However, to our knowledge, little is known about the importance of such regions in phorbol-dependent activation of PKC catalytic activity in the context of the entire enzyme. Moreover, there may be additional regions within and outside of the phorbol-binding regions that play critical roles in phorbol-dependent activation of PKC. Unlike experiments involving small fragments of PKC, studies using the holoenzyme permit the formation of secondary and tertiary structures, where mutagenesis of one region within PKC may have profound effects on the function of other regions. Consistent with this hypothesis, recent evidence suggests that the C1 and C2 regions of PKC cannot be considered independent membrane-binding modules.[34] Rather, tethering between the C1 and C2 domains of PKC appear to maintain PKC in an inactive conformation, and activation of the enzyme appears to take place through the ability of PKC activators to interfere with C1/C2 tethering.[36] Therefore, scanning mutagenesis studies involving the entire PKC holoenzyme may prove to be highly informative, particularly in identifying the consequences of mutagenesis of one region of the enzyme on the functionality of another region.

To better elucidate structure–function relationships for PKC and the role of the regulatory domain in PKC function, we constructed a library of tandem alanine-scanning mutations spanning the length of a V1-deficient human PKC.[37] By assessing the ability of these alanine-scanning mutant regulatory domains to inhibit PKC catalytic activity, we were able to identify regions within the PKC regulatory domain lying outside of the pseudosubstrate sequence that are important for inhibition of catalytic domain activity consistent with other studies.[33,38] By fusing each of these constructs in-frame with the entire PKC catalytic domain, we have been able to assess, in this study, the role of various regions within PKC for phorbol ester binding and for phorbol-dependent activation of the enzyme.

This study supports the importance of the C1b region for phorbol binding to the holoenzyme and identified "phorbol activation sites" that, when mutagenized, affect the ability of phorbol esters to activate catalytic activity *in vivo*. Only a fraction of these phorbol activation sites, when mutagenized, had a negative effect on phorbol binding. This study thus differentiates sites important for phorbol binding from sites important for phorbol-dependent activation of the enzyme. In addition, this study underscores the importance of C1/C2 interactions in PKC activation, and the importance of multiple regions within C2 for enabling phorbol esters to activate the enzyme.

Results

Expression of wild-type and mutant human PKCα holoenzymes in yeast

Each plasmid described in Figure 1 was used to transform yeast cells. In addition, constructs coding for the expression of glutathione-*S*-transferase (GST) (G), untagged PKCα (P) and untagged PKCα lacking the V1 region (PΔV1) were prepared, along with mutant GP-K368R and GPΔV1-K368R constructs, which render the enzyme catalytically inactive.[39,40] Yeast cells transformed with a yeast expression vector (pYES2) lacking a cDNA insert were prepared as a negative control. All constructs were confirmed to be correct by restriction endonuclease mapping and DNA sequencing. Six distinct yeast transformants were isolated per construct and grown on Ura^- plates containing 2% glucose. To induce expression of proteins by the constructs, clonal isolates housing each construct were grown in identical liquid medium containing 1% galactose. Whole cell lysates were prepared from an equal number of cells bearing the various constructs, and the lysates assessed for protein content by staining with Ponceau S red after electrophoresis or for expression of PKC-related proteins by immunoblotting experiments using an