The broad specificity of dominant inhibitory protein kinase C mutants inferences a common step in phosphorylation

Pilar GARCIA-PARAMIO, Yolanda CABRERIZO, Frederic BORNANCIN1 and Peter J. PARKER2

INTRODUCTION

Protein kinase (PK) C is a family of serine/threonine protein kinases that are regulated through agonist-induced hydrolysis of membrane phospholipids and are considered to play critical roles in many signal-transduction pathways [1–3]. This enzyme family comprises eleven members divided into three groups: (i) classical (c)PKC (α, β, γ, and δ), which are Ca+2- and phorbol-ester/diacylglycerol (DAG)-dependent; (ii) novel (n)PKC (ε, ζ, η, θ and ι/δ/KD), which are phorbol-ester/DAG-dependent but do not require Ca+2; and atypical (a)PKC (ε, ζ, η, θ and ι/δ/KD), which do not respond to Ca+2 or phorbol ester/DAG, although PKCζ can be activated by phosphatidylinositol 3,4,5-trisphosphate [4], as can other PKC family members [5–7].

Several reports have revealed an additional and highly efficient mode of control of PKC involving its phosphorylation. This may involve both autophosphorylation [8] and transphosphorylation [9,10], as well as activation-induced dephosphorylation [11,12]. Consistently with a critical role in function, PKCζ expression in bacteria yields an unphosphorylated precursor that does not require phosphorylation for the formation of a functional enzyme–substrate complex, but does appear to require phosphorylation for catalytic activity [13]. In eukaryotes, pulse–chase experiments demonstrate that PKCζ undergoes a series of phosphorylations within the first hour post-synthesis that account for its conversion into an active, albeit effector-dependent, protein kinase [14].

Cazaubon and colleagues [15,16] have shown that the phosphorylation of a threonine residue present in the activation loop within the catalytic domain is involved in controlling PKCζ activity. Similar observations have been made for PKCβ1 [17]. In PKCζ, the substitution of Thr-497 → Ala results in the production of an unphosphorylated and inactive 76 kDa PKCζ form. Co-expression in COS-1 cells of an activation loop PKCζ mutant and wild-type (wt) PKCζ leads to the expression of a fast migrating PKCζ1 protein that is similar in size to the unphosphorylated primary translation product of PKCζ [15]. Thus this PKCζ mutant appears to have dominant negative properties that can affect PKCβ1.

Recent studies have suggested that other relevant phosphorylations take place in PKCζ. Phosphorylation at Thr-638 at the C-terminus of PKCζ controls the duration of activation by regulating the rate of dephosphorylation and, consequently, inactivation of PKCζ [18]. The phosphorylation at the equivalent site of PKCβ1 (Thr-642) and PKCβ2 (Thr-641) may be essential for PKCβ activity [19,20], although these proteins may also be hypersensitive to dephosphorylation and inactivation (discussed in [14]). Further studies on PKCζ define the Ser-657 site of phosphorylation as controlling the accumulation of phosphate at other sites on PKC, as well as contributing to the maintenance of the phosphatase-resistant conformation [14]. Sites equivalent to Thr-638 and Ser-657 have been shown to be phosphorylated in baculovirus-expressed PKCζ/β1 [19,21]. Recent studies have shown that in PKCβ1, the C-terminal 642 site also controls Ca+2 affinity [22].

Although priming phosphorylations have been well-characterized in the cPKC isotypes PKCα and PKCζ, little is known of these events in the novel and atypical classes of PKC. In order to investigate priming phosphorylation in the non-conventional PKCs and the specificity of these processes, activation loop mutations were derived for novel and atypical PKCs. Here we show that putative phosphorylation-site mutation in the activation loops of PKCδ, PKCε and PKCζ confer dominant negative properties on these proteins in vitro. Furthermore, the results show that these inhibitory properties operate in a heterologous fashion, providing evidence for a common step in the processing of all these PKC isotypes.

MATERIALS AND METHODS

Construction of PKCζ, δ, ε and ζ mutant cDNA expression vectors

PKC mutants were constructed using bovine PKCζ, rat PKCδ, mouse PKCε and rat PKCζ cDNAs [23–26]. Exchange of the

Abbreviations used: DAG, diacylglycerol; FACS, fluorescence-activated cell sorting; PK, protein kinase; PKM, catalytic fragment of PKC; YSS, pseudo-substrate site; TRE, TPA (or PMA) response element; wt, wild-type.

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the p-Alt-1 vector and mutagenized with appropriate oligonucleotides to create the full coding sequence of each respective PKC were cloned into the Altered Sites mutagenesis system (Promega). Briefly, PKCδ EcoRI, PKCε Xhol and PKCζ Xhol fragments containing the full coding sequence of each respective PKC were cloned into the p-Alt-1 vector and mutagenized with appropriate oligonucleotides (Table 1). The introduction mutations created several new restriction sites: Haell in δ(T/A), ε(ST/A), and NotI in δ(ST/A), ε(T/A), and ε(T/A); these sites were utilized during the screening. The mutated sequence was verified by sequence analysis using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH, U.S.A.). The EcoRI digestion of the pA-alt-1 plasmids containing the different PKCδ mutants yielded a 2.8 kbp fragment that was subcloned into pcDNA3 for expression in COS-1 cells. The Spel/KpnI digestion fragment of pA-alt-1 containing the PKCε mutants was subcloned in the wrong sense into pSL301. From this vector, a 3.1 kbp KpnI/SstI fragment was subcloned into pcDNA3. PKCζ mutants were digested with SstI/EcoRI from pA-alt-1 and recloned using an EcoRI/Xhol fragment into pcDNA3. The PKCζ(T/A)ζ mutant was as described previously [15].

The constitutively active PKCζ and ε mutants were expressed using the pMT2 vector. These PKCζ are full-length z and z constructs with a deletion in their inhibitory pseudo-substrate sequences within the regulatory domain. Mutants in the regulatory domain were generated by deletion of amino acids 22–28 in the pseudo-substrate site (ψSS) of PKCζ [27] and 154–163 in PKCε (ψSS) [28]. The PKCζ regulatory domain was generated by taking the SacI fragment from pKS-1 PKCζ. The 3'-overhang was removed by T4 DNA polymerase and the fragment was cut with EcoRI at the 5'-end, followed by ligation into pcDNA3 restriction-digested with EcoRI/Xhol (blunted). For the ε mutant of the catalytic fragment of PKCζ (PKMζ; the PKMζ mutant), the BstXI/Xhol restriction fragment of PKCζ was subcloned into a BstXI/Xhol restriction-digested His6-tagged PKMζ (wild-type) construct (F. Bornancin and P. J. Parker, unpublished work). For the construction of the membrane-targeted CD2-PKM(T/A)ζ mutant, the catalytic domain of PKCζ(T/A)ζ (from bp 972 to the 3'-end) was amplified by PCR using the following oligonucleotide primers: 5’-GGCCTGCGCCGCAAGCTTGGC-CCTGGCGCAACA 3’-GTAGGAGCTTCGCGCCATAC-TGAGTCCGATCC. A NotI restriction site was introduced at the 5'-end, for ligation into pcDNA3/ BamHI-rCD2/NotI (kindly provided by Doreen Cantrell, ICRF, London) restriction-digested with NotI and Xhol.

To demonstrate that the newly generated construct CD2-PKM-ζ(T/A)ζ in pcDNA3 encoded the correct PKMζ fusion protein, the DNA was transiently transfected by electroporation into COS-1 cells. Western blot analysis with rCD2 mAb, Ox34, revealed the presence of the expected approx. 70 kDa species. Parallel with Western blot analysis, cell-surface expression of the CD2-PKMζ(T/A)ζ chimera was demonstrated by flow cytometry (or fluorescence-activated cell sorting; FACS), employing Ox34.

**Table 1 Oligonucleotides employed in the generation of the T-loop PKC mutants**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Changed aa</th>
<th>Mutagenic oligonucleotide</th>
<th>Screening oligonucleotide</th>
</tr>
</thead>
</table>
| δ(T/A) | 505         | AACCGGGGCGAGCGCTTTCTCGGCC | -s sense: 5’-GGGAGCTGCGCAATGAGC3’ |-
| δ(ST/A) | 504/505       | GAGAAAGGGGCGAGCGCTTTCTCGGCACT | antisense: 5’-TTACAGGAGCTTTACA3’ |-
| ε(T/A) | 566         | AATGGTGACAACTACGCTTCTGGG | -c sense: 5’-TGGGGAGCATTACCTCG3’ |-
| ε(T/A) | 563/564/556/556 | ATGAGGAGCTTGCGCCCTTCTGGGACT | antisense: 5’-ATGGAATGATCTACGG3’ |-
| ε(T/A) | 504         | CCCGGGACACAAAGCGCCTTTTGGA | -ς sense: 5’-GTCAAGGGGAGGGC3’ |-
| ε(T/A) | 410         | GGCCCGCCGAGCGCTGCCGCTTTTGTGAAACC | antisense: 5’-GGATCATCTACCGGCG3’ |-

**Tables**

**Table 1 Oligonucleotides employed in the generation of the T-loop PKC mutants**

The mutagenic oligonucleotides (with mismatches underlined), the changed amino acid (aa) residue numbers and the oligonucleotides employed for screening are shown for the PKCα, PKCε and PKCζ proteins.

COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% (v/v) foetal-calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C in 10% CO2 in humidified air. One day before transfection, COS-1 cells were plated at a density of 0.3 × 10⁶ cells per 60-mm culture dish. Calcium phosphate DNA precipitates were prepared with 5 µg of TRE-luciferase, 0.5 µg of the constitutively active mutant and 1 µg of each plasmid expressing an individual PKC-isotype mutant or the control vector pcDNA3 or 5 µg of TRE-luciferase plus 1.5 µg of empty vector. An equal amount of transfection mix was added to two separate plates for each transfected sample. In some experiments, these plates were harvested separately for luciferase assays and for electrophoresis and Western blot analysis. The cells were washed three times with Dulbecco’s modified Eagle’s medium 12–15 h after transfection, and then fresh culture medium with 2% (v/v) foetal-calf serum was added to the dishes. The dishes were washed twice with PBS 48 h after transfection. Then 300 µl of lysis buffer [0.65% Nonidet P40/10 mM TriS/1 mM EDTA/150 mM NaCl] was added; the lysate was transferred to a tube when the intact nuclei were visible under the microscope. After centrifugation, 20 µl of the supernatant was transferred to a polysulphon luminometer cuvette with 350 µl of luciferase reaction buffer [25 mM glycyl-glycine (pH 8.0)/5 mM ATP (pH 8.0)/15 mM MgSO4]. Luciferase activity was determined by addition of 33 µl of 3 mM Luciferin (Sigma) as described previously [30]. The background measurement was subtracted from each duplicate, and experimental values are expressed as the percentages of the total activity found in extracts from cells transfected with constitutively active mutants. Some of these plates were harvested with 200 µl of
Laemmli sample buffer [31] and samples were subjected to SDS/PAGE and Western blot analysis (see text and Figure legends).

**Western blot analysis**

COS-1 cells were transfected as above, except for the data in Figure 3 where 10% (v/v) foetal-calf serum was employed. After 48 h of transient expression, the cells were washed with ice-cold PBS and lysed in 300 µl of an ice-cold lysis buffer containing 20 mM Tris/HCl, pH 7.5, 10 mM EGTA, 10 mM EDTA, 1% (v/v) Triton X-100, 2 mM 2-mercaptoethanol, 5 mM benzamidine, 50 µg/ml leupeptin, 50 µg/ml aprotinin, 50 µg/ml trypsin inhibitor, 5 µg/ml pepstatin, 1 mM PMSF, 20 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 1 mM p-nitrophenyl phosphate. This procedure, and all others subsequently, were performed at 4°C. The suspension was subjected to 50 strokes in a Dounce homogenizer and centrifuged at 12000 r.p.m. (14000 g) for 30 min at 4°C. 100 µl of 4 × SDS sample buffer was added to the supernatant (Triton-soluble extract) and the pellet was resuspended in 400 µl of 1 × SDS sample buffer (insoluble fraction). Cell lysates were sonicated, boiled for 3 min, separated on a running gel containing 7.5% (v/v) acrylamide and 0.06% (v/v) bisacrylamide, and then subjected to immunoblot assay with polyclonal antibodies specific to each PKC as described previously [24,26,32,33].

**Other methods**

Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.), with BSA as the standard.

**RESULTS**

To assess the dominant negative potential of PKC activation loop phosphorylation-site mutants, two of these were tested for their ability to block reporter gene activation by their cognate activated forms, mutated at their inhibitory pseudo-substrate sites [27]. This latter mutation confers ligand-independent activity on the proteins, but does not by-pass any requirement for post-translational modification. As illustrated in Figure 1 (left panel), activated PKCα co-transfected with a TRE-luciferase construct induced an approx. 10-fold increase in luciferase activity, consistently with previous data [27,34,35]. Co-expression of the mutant PKCα(T/A)ε with activated PKCα leads to a complete block in luciferase induction. Thus this mutant PKCα protein has dominant negative properties with respect to PKCα.

In a similar fashion, the effect of two mutant PKCε constructs was tested on the ability of activated PKCε to induce luciferase from a TRE-dependent reporter construct. Unlike wtPKCε, wtPKCε overexpression alone is sufficient to elicit luciferase induction (Figure 1, right panel). A similar ‘basal’ activity of wtPKCε has been noted on transfection of this construct into RAW cells [35]. Expression of the activated PKCε produces a more effective (approx. further 2-fold) increase in luciferase. This induction is inhibited by both the (T/A)δ and (T/A)γ PKCε mutants (65% and 75% inhibition respectively), although their efficacy is less than that observed for the PKCδ mutant acting on PKCε.

The above data demonstrate that both the PKCα and PKCε mutants have dominant negative effects on their active counterparts. To determine the specificity of action of these mutants, as well as similar mutant forms of PKCβ and PKCζ, co-transfection experiments were carried out with activated PKCα, a TRE-luciferase reporter and each of the mutant PKC isotypes (or the appropriate vector control). As illustrated in Figure 2, all the mutants tested inhibit activated PKCα induction of luciferase. In each case, Western blot analyses were performed to determine (i) the expression of the dominant negative constructs for δ, α and ε (Figure 2, inset) and (ii) the expression of the activated PKCα (lower panel). Although each mutant was expressed, evidently PKCε(T/A)ε and PKCζ(T/A)ζ were poorly expressed compared with their (T/A)α counterparts (there was no consistent difference in the electrophoretic migration of the mutants of an individual isotype). Nevertheless, these (T/A)α mutant constructs proved to be more effective as inhibitors than the (T/A)δ, mutants. Table 2 presents a summary of the inhibitory effects of these constructs on luciferase induction by activated PKCα and activated PKCε.

Over the entire series of experiments, all dominant negative mutants showed a mean inhibition of at least 50%. However, there are relative differences: thus PKCε(T/A)ε is a more effective inhibitor than PKCζ(T/A)ζ of activated PKCα, whereas the reverse is true for activated PKCε. Similarly, PKCζ(T/A)ζ is a poorer inhibitor of activated PKCζ than PKCδ(T/A)δ, whereas the opposite is true for activated PKCε. The data indicate that these PKC mutants are not specific for the individual isotypes, although they do display relative differences in specificity.

The Western blots of activated PKCα (Figure 2) provide evidence that all the mutants modulate PKCα phosphorylation. This is consistent with previous studies on the PKCα(T/A)δ mutant [15]. The active phosphorylated form of PKCα is the 80 kDa species and it is evident from the data shown that all mutants reduce the amount of this form in a manner that approximately corresponds to their ability to inhibit luciferase induction. Thus the weakest inhibitor in this series of experiments [PKCζ(T/A)ζ] also causes the smallest loss of the 80 kDa PKCα species. These shifts in the 80 kDa species of activated PKCα are consistent with inhibitory effects of the mutant PKC isotypes on phosphorylation. However, owing to the constitutive accumulation of dephosphorylated, activated PKCα, it is impossible to assess increased accumulation of this dephosphorylated form (ligand-activated, open-conformation PKCα has been shown to be a much better substrate for protein phosphatases than the
Figure 2 Inhibition of constitutively active PKCα-induced TRE-luciferase activity by dominant negative mutants of PKCδ, PKCε, and PKCζ

COS-1 cells were transfected with a TRE-luciferase reporter construct together with either an empty vector (white) or with the PKCα constitutively active, α✻ (black) or the PKCα constitutively active together with the different mutants for PKC-δ, -ε, and -ζ (dotted). The induced luciferase activity is shown in arbitrary units (au). This is one of seven independent experiments (see summary of data in Table 2). The inset is a Western blot showing the expression of the dominant negative mutants.

Table 2 Inhibition of constitutively active PKCα or PKCε by different PKC dominant negative mutants

The experiments were carried out as indicated in Figure 2. The data shown are the mean for seven independent transfections, each performed in duplicate. The luciferase activity is expressed as a percentage of the luciferase activity induced by each constitutively active mutant alone after subtraction of the activity induced by the empty vector.

<table>
<thead>
<tr>
<th>Dominant negative mutants</th>
<th>Activated mutants</th>
<th>Luciferase activity (% of the control)</th>
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<tbody>
<tr>
<td></td>
<td>α✻</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>ε✻</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>Vector</td>
<td></td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>α(T/A)3</td>
<td></td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>δ(T/A)3</td>
<td></td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>ε(T/A)4</td>
<td></td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>γ(T/A)α</td>
<td></td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>ζ(T/A)β</td>
<td></td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>ζ(T/A)γ</td>
<td></td>
<td>100.0 ± 0.0</td>
</tr>
</tbody>
</table>

inactive protein [11], consistent with the observed accumulation of the dephosphorylated activated form. In order to determine the effect on dephosphorylation in a more suitable context, dominant negative mutants were tested on wtPKCα, which accumulates largely as an 80 kDa species (i.e. fully phosphorylated). To evaluate the effects of the mutants on the phosphorylation state of wtPKCα, co-transfections were carried out as described above for the constitutively activated forms. As shown in Figure 3, each of the mutants leads to the accumulation of dephosphorylated wtPKCα and a substantial proportion of this dephosphorylated protein is characteristically in the Triton X-100-insoluble fraction. At these ratios of wt:mutant PKC plasmid, the inhibitory effect is incomplete: at higher ratios, the inhibition can be virtually complete (for example, see [15]); the data shown here reflect the concentrations employed with the activated PKC isotypes. For PKCα(T/A)α, longer exposure of the Western blot was required to reveal the insoluble PKCα that accumulated; this is consistent with the weaker effect of this mutant (see Table 2).

The broad specificity of these dominant negative PKC mutants and their action indicates that they might each suppress phosphorylation by a non-productive interaction with a shared binding protein (e.g. a common PKC kinase). However, it is possible that inhibition is exerted through regulatory domain interactions with effectors, in the context of compromised protein kinase
activity. To test this, a PKCζ regulatory domain construct was co-expressed with wtPKCζ. However, this deletion mutant had no effect on PKCζ phosphorylation, as judged by its migration at 80 kDa (Figure 4A). In order to assess the minimum requirement for inhibition, the kinase domain of PKCζ containing the (T/A)₃ mutation was also investigated. This construct was found not to inhibit PKCζ phosphorylation (Figure 4). Furthermore, membrane targeting of the PKCζ(T/A)₃ as a CD2-fusion construct was also found not to inhibit wtPKCζ phosphorylation (Figure 4B), despite its expression at the membrane as determined by FACS analysis (results not shown). The results indicate that effective competition requires an intact mutant kinase.

**DISCUSSION**

The results presented here have a number of implications for PKC function and properties. It is established that mutations in the activation loops of PKCζ, ε and ζ have dominant negative properties on expression in COS-1 cells. It is shown also that these dominant negative properties operate in a heterologous fashion, e.g. PKCζ(T/A)₃ inhibits activated PKCζ. These dominant negative mutants act by blocking the accumulation of phosphorylated PKC, as evidenced by studies with PKCζ, indicating that there is a common titratable component that promotes the priming phosphorylations of all these PKC isotypes. Furthermore, this competitive interaction is shown to require an intact mutant protein and is not supported by either the regulatory or mutant catalytic domains alone. It is concluded that phosphorylation of the wtPKCs requires appropriate localization and is effected through at least one overlapping step.

Previous studies from this laboratory have defined Thr-497 as a critical site of phosphorylation for PKCζ [16]. Similar data have also been reported for PKCζ [17]. The Thr-497 site resides in the predicted activation loop of PKCζ between kinase domains VII and VIII; the equivalent region is found to be phosphorylated in many other protein kinases [36]. Expression of ePKC isotypes in bacteria has led to the idea that phosphorylation in this region requires some other mammalian protein, the simplest interpretation being the action of a ‘PKC-kinase’. The finding that a PKCζ mutant modified in the activation loop has dominant properties was consistent with the view of competition with such a saturable ‘PKC-kinase’. Here we investigated the behaviour of cPKC, nPKC and aPKC isotypes mutated in this region. Like the original PKCζ mutant, it is established that all have dominant negative properties and that this dominance shows no absolute specificity towards particular PKC isotypes. Thus both activated cPKC and nPKC isotypes are functionally inhibited by mutants derived from all three (cPKC, nPKC and aPKC) subclasses. It is concluded that phosphorylation of all these PKC isotypes in their activation loops occurs in *vivo*, accounting for the observed behaviour of the mutants. Evidence derived from the use of site-specific antisera on PKC isotypes expressed in eukaryotic cells indicates that this is indeed the case (P. J. Parker, unpublished work). Thus although it has been argued that activation loop (Thr-505) phosphorylation of nPKCζ is not required for activity [37], eukaryotes nevertheless phosphorylate this site.

These results clearly demonstrate that this type of mutant cannot be employed to define PKC isotype specificity in *vivo*. More interestingly, however, this lack of specificity defines a common step in the processing/activation of all these PKC isotypes. The simplest conclusion is that the mutants compete with a PKC kinase; however, inhibition could be effected at either a prior or parallel step. Studies on PKCζ phosphorylation indicate that all these mutants are able to inhibit the normal accumulation of a fully phosphorylated PKCζ protein (Thr-497, Thr-638 and Ser-657 sites [14]), coincidentally with an increased recovery of PKCζ in a Triton-X-100-insoluble fraction. This insolubility is characteristic of the accumulated dephosphorylated form, which is derived normally from activation-induced dephosphorylation (discussed in [14]). It has been observed that there is a distinction between the behaviour of the low concentration of unphosphorylated PKCζ primary translation product, which is soluble, and the dephosphorylated protein, which is insoluble. This distinction may reflect the normal operation of a PKCζ chaperone that binds to the primary translation product and is needed to maintain conformation, and hence solubility, before the protein’s phosphorylation [14]. In light of the observations here, it is plausible that the common saturable step in the processing of all these PKC isotypes is at the level of such a chaperone, i.e. it is this step that is blocked by the action of the dominant negative mutants. It is noteworthy that there are subtle differences in efficacy between these PKC mutants acting on different activated PKC isotypes. Thus although a common kinase and/or chaperone requirement may be involved in the priming phosphorylations, it is possible that this requires not one, but a series, of related proteins that have overlapping specificity but differing affinities for the PKC isotypes. Characterization of these priming events and the identity of the kinase(s) involved will resolve this issue.

Although the possible action of a common PKC kinase or PKC chaperone would account for the dominant negative activity of these PKC mutants, competition for lipid effector should be...
considered, since there is evidence that some part of these priming phosphorylations may be due to autophosphorylation [8]. In principle, competition for the allosteric activator DAG would block activation-dependent phosphorylation. However, while this might account for the cPKC and nPKC effects, there is much evidence that aPKCζ is not regulated by DAG (see, for example, [26]). It is therefore very unlikely that competition for DAG accounts for the effects of these proteins. This conclusion is substantiated by the finding that the PKCζ regulatory domain does not affect wtPKCζ accumulation as a fully phosphorylated form. The observation that a catalytic domain (T/A)ζ mutant, soluble or membrane-targeted, also fails to behave as PKCζ(T/A)ζ indicates that the effective inhibitory properties of these mutants requires an intact protein. This is likely to reflect isomeric constraints on the action of these dominant negative constructs.

Irrespective of the precise competing step in the processing of these PKCζ isotypes, the evidence here demonstrates that there is an inhibition at the level of the priming phosphorylations. This highlights the requirement for these phosphorylations in the normal operation of PKC. In particular, it is concluded that the conserved threonine within the sequence Thr-Phe-Cys-Gly-Thr present in the activation loops of PKCζ, -α, -ε and -ζ plays an essential role in maintaining the proteins in their fully primed, active (if latent) conformations. The inability to complete the phosphorylation of one of these proteins and produce the active (if latent) form is what is responsible for conferring dominant negative properties on the overexpressed mutants.

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