ABSTRACT

Title of Dissertation: NOVEL-TYPE PROTEIN KINASE C ISOFORM-SPECIFIC FUNCTION, KINASE ACTIVITY, AND PHOSPHORYLATION STATUS ARE DISRUPTED BY V5 DOMAIN MUTATIONS

Walter Antonio Schlapkohl, Doctor of Philosophy, 2005

Dissertation directed by: Professor J. Frederic Mushinski
Molecular and Cell Biology Program

The Protein Kinase C (PKC) family consists of at least 10 different isoforms that are subdivided into three groups: "classical/conventional" cPKCs (α, βI-II and γ), "novel" nPKCs (δ, ϵ, η and θ) and "atypical" aPKCs (ζ and λ/ι). The nPKCs are "novel" because they do not respond to changes in calcium levels as do the classical PKCs, but respond only to changes in diacylglycerol levels. This work elucidates several aspects of the V5 domain's role in nPKC function through the use of PKC-δ and -ϵ truncation mutants, as well as two reciprocal PKC-δ and -ϵ C-terminal chimeras: PKC-δ/ϵV5 and PKC-ϵ/δV5. First, the ability of the V5 domain to confer isoform-specific function was tested by overexpressing chimeric PKC mutants in 32D cells. Overexpression of wild-type PKC-δ, but not PKC-ϵ, mediated macrophage differentiation in this cell line upon stimulation with TPA. However, neither PKC-δ/ϵV5 nor -ϵ/δV5 were able to confer a TPA-inducible macrophage differentiation phenotype to 32D cells, indicating that PKC-δ V5 domain was essential to this

isoform-specific function, but not sufficient to transfer its activity to PKC-ε. Second, nPKC mutants were utilized to determine the ability of the V5 domain to modulate kinase activity. PKC chimeric and truncation mutants had significantly decreased kinase activity *in vitro* compared to their wild-type counterparts. PKC-δ/εV5 and -ε/δV5 lacked phosphorylation at critical phosphorylation priming sites, a likely cause of their reduced kinase activity. Glutamic acid substitutions made at unphosphorylated priming sites, either individually or in combination, did not rescue kinase activity. Stimulation with TPA increased the level of phosphorylation only at sites that were already phosphorylated. The lack of phosphorylation and kinase activity are likely due to differences in phosphatase activity, since both PKC-δ and -ε V5 chimeras bound the upstream, activating kinase, Phosphoinositide-dependent Kinase-1. In conclusion, changes to the V5 domain of PKC-δ and -ε disrupted the proteins' phosphorylation status and kinase function which, in turn, likely prevented conferral of isoform-specific function in a macrophage differentiation assay with PKC-δ and -ε V5 chimeras.

NOVEL-TYPE PROTEIN KINASE C ISOFORM-SPECIFIC FUNCTION, KINASE ACTIVITY, AND PHOSPHORYLATION STATUS ARE DISRUPTED BY V5 DOMAIN MUTATIONS

By

Walter Antonio Schlapkohl

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Advisory Committee:

Professor J. Frederic Mushinski, Chair
Professor Albert Ades
Professor Kevin Ridge
Professor Wenxia Song
Professor Stephen Wolniak
Professor William Higgins, Dean's Representative

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ABBREVIATIONS

AKAPs…………………....A-Kinase Anchoring Proteins

ASIP…………………….atypical PKC isotype specific interacting protein

aPKC……………………atypical Protein Kinase C

cPKC……………………conventional/classical Protein Kinase C

DAG………………….....diacylglycerol

F…………………………forward primer

GFP……………………….green fluorescent protein

HA………………………...haemagglutinin

IP_3……………………….inositol 1,4,5-trisphosphate

mTOR ……………………mammalian Target of Rapamycin

nPKC……………………..novel Protein Kinase C

PDK1……………………..Phosphoinositide-dependent protein kinase 1

PIP_2………………………phosphatidylinositol 4,5-bisphosphate

PKC……………………….Protein Kinase C

R………………………….reverse primer

STICKs……………………Substrates that Interact with C-Kinases

TPA………………………12-O-tetradecanoylphorbol-13-acetate

INTRODUCTION

PKC Structure

The Protein Kinase C (PKC) enzymes are a family of related serine/threonine

protein kinases within the AGC Kinase superfamily that are important for the

transduction of numerous cell signals from the plasma membrane to the nucleus of the

cell. PKCs are also responsible for coordinating various signaling pathways,

modulating gene expression, and inducing cytoskeletal changes and other cell

biological processes. PKC expression and activation have been linked to cell

proliferation, differentiation, adhesion, cell cycles and other cell functions. Not

surprisingly, PKC plays an important role in cancer biology.

There are at least 10 structurally related isoenzymes of PKC grouped into three

separate classes based upon the structure of their regulatory domains: the conventional

or classical PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, η, and θ) and atypical PKCs

(λ/ι and ζ). All PKC isoforms require phosphatidylserine (PS) for optimal activity.

Conventional PKCs (cPKCs) can be activated by calcium and/or by diacylglycerol

(DAG) and phorbol esters. Novel PKCs (nPKCs) are not regulated by calcium but can

be activated by DAG and phorbol esters. Finally, atypical PKCs (aPKCs) are

unresponsive to calcium as well as to DAG and phorbol esters.

All isoforms of PKC have constant (C) and variable (V) domains (Figure 1).
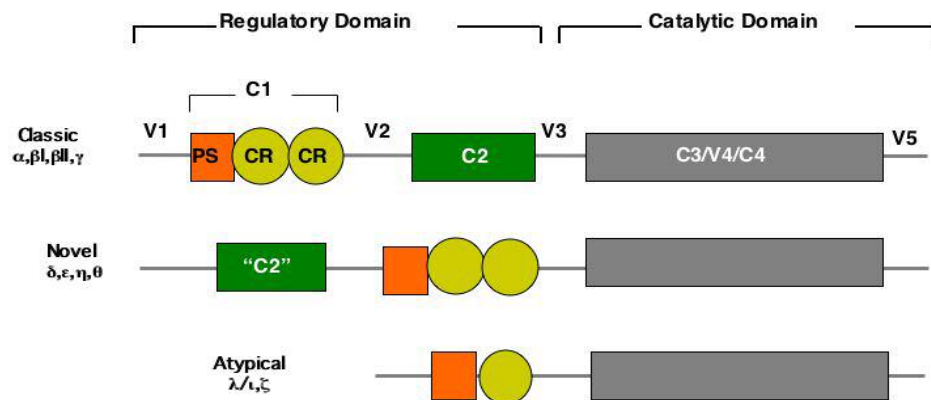
Figure 1. Structure of Protein Kinase C Isoforms. Constant domains (C1-C4) and variable domains (V1-V5) are shown. The phorbol esters and the second messenger DAG bind to the cysteine-rich (CR) motif present in cPKCs and nPKCs. C2 domains bind calcium except for nPKCs which have a modified ("C2") domain; PS, pseudosubstrate sequence; C3, ATP-binding domain; C4, kinase domain. The C3, V4 and C4 domains are often combined into one large kinase domain which defines PKC members within the AGC kinase superfamily.

Each of the constant domains of PKC has at least one known function. Indeed, more is known about those domains that all PKCs have in common than about what makes each isoenzyme unique.   Each PKC isoform has an amino(N)-terminal regulatory domain and a carboxyl(C)-terminal catalytic domain. The first isozymes were cloned in the mid-1980s and their sequences revealed four conserved domains: C1-C4 [1]. The C1 domain contains cysteine-rich motifs that serve as sites for DAG and phorbol ester binding [2]. The C2 domain allows conventional PKCs to bind to phospholipids in a calcium-dependent manner [3]. Novel PKC isoforms have a modified C2 domain that does not bind calcium. Very recent work by Stahelin $et\ al.$ has shown that PKC-δ's C2 domain does not bind to phospholipids, either [4]. Instead, it appears that the C2 domain of PKC-δ is a phosphotyrosine binding domain [5]. The C3 and C4 domains of the catalytic region of the protein comprise the ATP-binding site and the substrate binding site, respectively [6]. The C3, V4 and C4 domains are often combined and increasingly considered as one large kinase domain that defines PKC members within the AGC kinase superfamily. The regulatory and catalytic domains are connected by a hinge region (V3) that is subject to proteolysis when the enzyme becomes membrane-bound [7].

PKC Activity and Regulation

PKC transduces a myriad of signals that begin with lipid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). The dogma has been that the generation of DAG at the plasma

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membrane recruits PKC from the cytosol and induces an allosteric change in the its conformation, opening up the enzyme to permit substrate binding and signaling to the next level [8]. While this scheme may be generally true for the classical PKCs, there is substantial evidence that indicates that the mechanism of PKC activation is far more complex and that novel PKC activation and regulation may work quite differently from classical PKC activation [9, 10].

It is believed that all PKC isoforms are rendered mature only after a series of priming phosphorylations. Our knowledge of these priming phosphorylations has been derived in large part from studies of their function in classical PKC isotypes [11-13]. These priming phosphorylations occur at three conserved sites within the catalytic domain. Two of the three sites are found within the V5 domain. The first, and perhaps most critical, priming site is at the activation loop (Figure 2). Phosphorylation at this site aligns and stabilizes the two lobes of the kinase domain in preparation for catalysis [14]. Mutations to the activation loop site profoundly block classical PKC activities [15, 16]. However, unlike classical PKCs, the novel PKC-δ does not require this phosphorylation for catalytic competence, although catalytic activity is reduced when the activation loop is not phosphorylated [17].

Phosphoinositide-dependent protein kinase 1 (PDK-1) has been shown to phosphorylate classical, novel as well as atypical PKCs at the activation loop site [10, 18]. Accumulating evidence shows that in mammalian cells PKCs are constitutively phosphorylated at the activation loop, although this site can become dephosphorylated in PKC-δ and -ε in quiescent, serum-starved cells. In this case phosphorylation
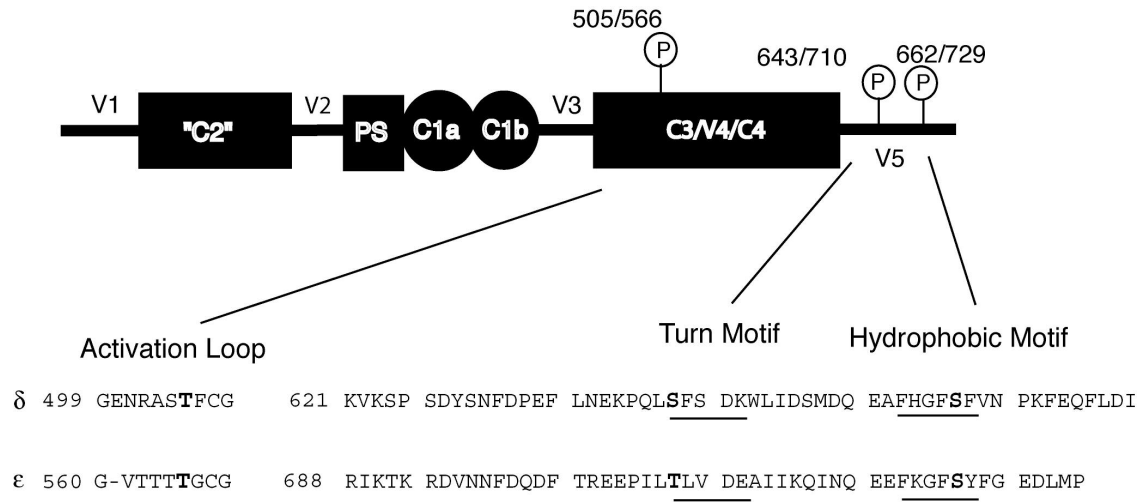
Figure 2. Alignment of activation loop, turn motif and hydrophobic motif phosphorylation sites in murine PKC-δ and -ε. The schema of Protein Kinase C on top shows the relative positions of the phosphorylations for PKC-δ and -ε respectively. Phosphorylation sites are in bold and their respective motifs are underlined.

at this site quickly occurred upon stimulation with serum or platelet-derived growth factor [10, 19].

Phosphorylation at the activation loop leads to rapid phosphorylation at the second priming site within the proline-rich "turn motif" (Figure 2). This is an autophosphorylation site for conventional PKC isoforms [20] as well as the novel isoforms PKC-δ [21] and -ε [10]. This phosphorylation site is thought to lie at the apex of a turn on the upper lobe of the kinase domain. Phosphorylation of the turn motif appears to lock the enzyme into a stable, catalytically competent conformation that is resistant to phosphatase activity [12, 22]. The known 3D structure of PKC's cousin, PKA, suggests that the phosphorylated residue at this site serves to anchor the V5 domain to the upper lobe of the kinase domain [23].

The mechanism of phosphorylation at the final priming site, the hydrophobic motif site (Figure 2), seems to be dependent upon the isoform. For classical isoforms the final priming step is again an autophosphorylation event. However, the same may or may not be true of novel PKC-δ and -ε. Work by Cenni *et al.* has indicated that the hydrophobic motif priming site in PKC-ε (S729) might be an autophosphorylation site based on the following evidence: first, wild-type PKC-ε overexpressed in HEK 293 cells was phosphorylated at this site but a kinase-deficient PKC-ε construct was not; second, PKC-ε was phosphorylated at S729 in the absence of any other kinase in an *in vitro* kinase assay; third, phosphorylation at this site was reduced in cells incubated with a novel PKC inhibitor [10].

However, other groups are casting light on what may be a much more context-dependent phenomenon. Rybin *et al.* have used cardiac myocytes to demonstrate a

dynamic and tightly-controlled regulation of phosphorylation within the hydrophobic motif of PKC-ε that appears to be under the control of PKC-δ [24]. Parker and his colleagues have shown that the hydrophobic motif phosphorylation site on PKC-δ is regulated by a heterologous kinase, perhaps PKC-ζ [25], in 293 cells. In a separate study by the same group, phosphorylation at the hydrophobic motif of PKC-δ and -ε was shown to be downstream of mTOR (mammalian Target of Rapamycin) on an amino acid sensing pathway [19]. Regardless of the mechanism used, this phosphorylation site appears to remain exposed until it becomes phosphorylated, and it serves as a docking site for PDK-1 in the unphosphorylated state [26]. Once phosphorylated and buried, the hydrophobic motif contributes to the stability of the active enzyme [3].

Once mature, PKC is ready to respond to changes in cofactor concentrations. Increases in DAG or phorbol ester levels recruit novel PKC molecules to membranes where they undergo an allosteric change that releases the pseudosubstrate domain from the catalytic half of the protein. This open conformation is then free to bind to co-localized substrates. Once bound to its target, PKC phosphorylates serine and threonine residues, usually within basic sequences. A very short consensus sequence has been deduced: S/T-X-K/R [27] where X is any amino acid. In some cases two amino acids appear between the critical phosphorylation site and the conserved lysine or arginine residue.

In addition to the translocation induced by changes in second messenger levels and (in the case of at least novel PKCs) the dynamic regulation of the priming phosphorylations, PKC activity is also regulated by a battery of binding partners and

anchoring proteins [28]. These proteins can act as scaffolds, positioning PKC near the proper substrates. They may also act as anchors, keeping PKC in place in long enough to co-localize with regulators such as other kinases, phosphatases or organelle-specific proteins. A large variety of PKC-binding proteins have already been identified: STICKs (Substrates that Interact with C-Kinases), cytoskeletal proteins such as actin and tubulin, scaffolding proteins such as AKAPs (A-Kinase Anchoring Proteins) and certain caveolin isoforms as well as RACKs (Receptors of Activated C-Kinases) [9]. To date, RACKs have only been identified for the PKC-β, -δ and -ϵ isoforms, and in each case the respective RACK protein is known to serve other functions in addition to PKC binding [29-31].

Biologically, PKCs play a role in receptor desensitization [32], transcriptional regulation [33], the modulation of membrane structure events [34], and the regulation of cell proliferation [35] and differentiation [36], among other things. Phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) have often been utilized to stimulate such PKC-related effects. For example, the human myeloid cell lines, HL-60 and U937, stopped dividing and differentiated along the monocytic lineage when stimulated by TPA [37, 38], whereas TPA stimulation increased proliferation in human SNU-C1 and SNU-C4 colon cancer cell lines [39]. PKC activation was implicated in these studies, since it is the major intracellular receptor for phorbol esters [40].

Yet the lack of a means to directly detect activation of specific PKC isozymes *in vivo* stands as a large roadblock in evaluating the role of individual PKC isozymes in such systems. To study isozyme-specific effects upon differentiation and proliferation, one can examine the subcellular localization of isozymes upon differentiation or

introduce kinase-defective isoforms as dominant-negative mutants. Overexpression of specific PKC isoforms can also be utilized to identify isoform-specific function. For example, CHO cells that overexpress PKC-δ reduced their growth rate in the absence of TPA and were arrested in the G2/M phase in the presence of TPA [41]. In contrast PKC-ε overexpression in rat fibroblasts induced evidence of neoplastic transformation, such as anchorage-independence and increased cell proliferation [42]. Although examples can be found to the contrary, the literature generally has borne out a "differentiating" or "apoptotic" role for PKC-δ and a "proliferative" or "oncogenic" role for PKC-ε.

Experiments conducted in the Mushinski lab with these isoforms are in agreement with this isoform-specific phenotypic pattern. In 32D cells, a mouse promyeloid cell line, overexpression of PKC-α or PKC-δ resulted in macrophage-like differentiation in response to TPA stimulation, whereas overexpression of PKC-βII, -ε, -η and -ζ with TPA stimulation did not [36]. The differentiation of 32D cells was assessed by several criteria: morphology, surface adherence, lysozyme production, pahgocytosis and macrophage-specific cell surface markers. Specifically, TPA treatment of 32D cells overexpressing PKC-δ dramatically increased cell volume, induced surface adherence in roughly 25-33% of the cells and resulted in Mac-1 and Mac-3 expression at the cell surface. Mac-1 (CD11b, CD18) is predominantly expressed on myeloid and natural killer cells, where it mediates numerous physiological functions, including phagocytosis of foreign particles, migration and adhesion of leukocytes to the endothelium and activation of neutrophils and monocytes [43]. Mac-3 is also involved in cell adhesion and serves as a marker for peritoneal

macrophages [44]. In contrast, overexpression of PKC-ε in NIH 3T3 cells stimulated cell proliferation, increased growth to saturation density and induced anchorage-independent growth. These overexpressing cells grew as tumors in nude mice [35, 42].

Examples of TPA-stimulated myeloid cell differentiation are certainly not limited to 32D cells. Normal mouse peritoneal monocytes and the human cell lines HL-60 and U937 are able to differentiate upon TPA stimulation alone [36]. These cells generally express easily detectable levels of PKC-α and -β and low levels of PKC-δ and -η, yet it remains unknown whether a specific PKC isoform is responsible for the TPA-induced differentiation. In contrast, 32D cells express endogenous PKC-α, -δ, -η and -μ. Overexpression of PKC-δ in these cells seems to upregulate endogenous PKC-δ expression, whereas overexpression of PKC-ε expression downregulates endogenous PKC-δ expression (data not shown). Other changes in endogenous PKC expression resulting from overexpression of PKC-δ or -ε in 32D cells have not been tested.

Lessons from PKC Constructs and Mutants

The oncogenic and differentiating potential of overexpressed PKC-ε and -δ has been mapped to their respective catalytic domains with chimeric mutants that consist of the regulatory domain of one isoform fused to the catalytic domain of the other. Experiments with these chimeras, designated PKC-δε and -εδ, revealed that the catalytic domain of PKC-ε imparted oncogenic characteristics to the protein when overexpressed in NIH 3T3 cells activated by TPA stimulation [45]. Fibroblasts that overexpressed the PKC-δε chimera showed decreased doubling time and the ability to

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grow in soft agar [45]. The overexpressed PKC-εδ chimera retarded cell growth and mediated phorbol ester-induced differentiation of promyelocytes into macrophages [46].

Lessons from these chimeras were not limited to growth and differentiation assays. Translocation studies revealed that, in the absence of TPA, overexpressed PKC-ε and PKC-δε in NIH 3T3 cells localized mainly in the cytoplasm, although immunostaining showed some PKC-ε and δε in and on the nucleus [45]. Upon TPA stimulation both overexpressed enzymes translocated mainly to the plasma membrane, while the staining on and in the nucleus remained unchanged [45]. The same study showed that prior to stimulation, PKC-δ and -εδ were present mostly in the perinuclear region, and, after one hour of treatment with TPA, both isoforms were found on/in the nucleus.

That the subcellular localization and the translocation patterns of PKC-εδ and -δε were similar to that of PKC-δ and -ε, respectively, is likely not a coincidence. Because the PKC substrate consensus sequence is not very specific, PKC isoform specificity is achieved by restricting PKC isoforms to substrates within certain subcellular locales. As already mentioned, any number of PKC binding partners may assist in isoform-specific localization. For example, p32 (gC1qBP) has been identified as a RACK for PKC-δ, localizing it to the perinuclear region upon TPA stimulation [31]. p32's exact function is not known, but it is involved in mitochondrial function [47, 48], transcription [49] and splicing factor modulation [50]. The beta-coatamer protein β-COP' has been identified as a RACK for PKC-ε, localizing PKC-ε to Golgi vesicles where it is involved in Golgi budding and vesicular trafficking [51]. These

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binding partners may account for at least part of the translocation patterns observed in NIH 3T3 cells overexpressing these isoforms. P32 specifically may play an important role in PKC-δ's isoform-specific function, since it may be preferentially drawing PKC-δ to the perinuclear region in this context. However, it is not clear that PKC-δ localization to the nuclear membrane is required for 32D macrophage differentiation, and p32's known involvement in mitochondrial function or splicing do not indicate what kind of a role, if any, the p32-PKC-δ complex may play in this cellular assay.

V5 Domain Studies

The size of the PKC V5 domain has not been established functionally for the novel PKCs. The original PKC domain boundaries were established by Coussens *et al.* in 1986 when only three conventional isoforms of PKC were known [1]. At that time the V5 domain was considered to consist of approximately the most C-terminal seven amino acids, depending upon the isoform. Sequence comparison of all ten PKC isoforms has generally fit the original loose description of constant and variable regions, and the nomenclature has persisted. Functionally, however, it has been shown that deletion of the last 15 amino acids of the V5 domain of PKC-α, expressed in yeast, rendered the enzyme biologically inactive *in vivo* and catalytically inactive *in vitro* [52]. However, a PKCα/βIIV5 chimera was then constructed in which the most C-terminal 73 amino acids of PKC-βII replaced the final 77 amino acids of PKC-α. This chimera was found to restore PKC activity in yeast as demonstrated by increased calcium uptake in response to TPA stimulation and modulation of cell doubling time

by TPA [53]. A similar chimeric construct was found to translocate to the nucleus and phosphorylate lamin B – functions specific to PKC-βII [54].

Differences between the PKC isoforms βI and βII also provide insight into the size and function of the V5 region. The two isoforms are splice variants that differ only in the V5 region (here defined as the carboxyl most 50-52 amino acids). PKC-βI and βII translocate to distinct subcellular locations when activated in the same cell type [55, 56]. In addition, Blobe *et al.* showed that PKC-βII, but not -βI, specifically bound to actin upon TPA treatment [55]. Goodnight *et al.* showed co-localization of PKC-βII with actin upon TPA stimulation, while PKC-βI translocated from the cytoplasm to the plasma membrane upon similar treatment [56]. These studies and others have shown that the V5 domain of PKC-βI and -βII is associated with different physiological functions [57]. Consistent with such data, Staudinger *et al.* have shown that in PKC-α, the V5 domain contains a PDZ-binding domain which allows it to bind PICK1 protein [58]. PDZ domains are protein-protein interaction motifs that serve to localize proteins to specific subcellular sites [59-61] and have been implicated in PKC signaling pathways related to phototransduction [62]. PKC interacts with PDK-1 through binding with the hydrophobic motif in the V5 domain [63]. In addition, proteins and complexes, including mTOR, atypical PKC isotype specific interacting protein (ASIP) and PKC-ζ have been shown to bind to the V5 domain of one PKC isoform or another.

The present body of work seeks to determine the role of the V5 region in PKC-δ and -ε maturation, activation and biological function. Because previous V5 region studies have focused mainly on conventional isoforms, little is known about the function of the V5 region in the novel isoforms δ and ε. Nevertheless, much is known

about the role of PKC-δ and -ε in cellular function, and studies with PKC-δε and -εδ regulatory/catalytic chimeras have indicated that isoform-specific function was conferred by the catalytic half of the protein which includes the V5 domain [45, 46]. Moreover, two out of three critical priming phosphorylation sites have been mapped to this domain [64], yet their role in PKC function remains an area of active debate. Therefore the results of this study fill a gap in our knowledge of the role of PKC variable domains and provide a functional definition for the novel PKC V5 region. The findings from the experiments reported here were designed to elucidate the role of the V5 structural domain in conferring isozyme specificity. However, the results shed light on the role of the V5 domain in broader aspects of nPKC function and regulation, including kinase activity and post-translational modification.

Table 1 lists constructs made for use in determining the role of the V5 domain in PKC function. Not all constructs were used and not all those constructs used were included in every experiment.

Table 1. A list of constructs used in this project and a short description of each.

PKC Construct	Tag	Description
PKC-δ	GFP or HA	Wild type
PKC-ε	GFP or HA	Wild type
PKC-δ/εV5	GFP or HA	PKC-δ w/ V5 domain exchanged with that of PKC-ε
PKC-ε/δV5	GFP or HA	PKC-ε w/ V5 domain exchanged with that of PKC-∂
PKC-δ CD11	GFP or HA	PKC-δ w/ C-terminal deletion of 11 amino acids
PKC-δ CD24	GFP or HA	PKC-δ w/ C-terminal deletion of 24 amino acids
PKC-δ CD54	GFP or HA	PKC-δ w/ C-terminal deletion of 54 amino acids
PKC-ε CD6	HA	PKC-ε w/ C-terminal deletion of 6 amino acids
PKC-ε CD20	GFP or HA	PKC-ε w/ C-terminal deletion of 20 amino acids
PKC-ε CD50	HA	PKC-ε w/ C-terminal deletion of 50 amino acids
PKC-δ/ε23	GFP or HA	PKC-δ w/ terminal exon PKC-δ exchanged with that of PKC-ε
PKC-ε/δ18	GFP or HA	PKC-ε w/ terminal exon PKC-ε exchanged with that of PKC-δ
PKC-δ T505E	GFP	PKC-δ activation loop mutant (ALM)
PKC-δ S643E	GFP	PKC-δ V5 turn motif mutant (TMM)
PKC-δ S662E	GFP	PKC-δ V5 hydrophobic motif mutant (HMM)
PKC-δ DM	GFP	PKC-δ ALM + HMM double mutant
PKC-δ TM	GFP	PKC-δ ALM + TMM + HMM triple mutant
PKC-ε T566E	GFP	PKC-ε activation loop mutant
PKC-ε T710E	GFP	PKC-ε activation loop mutant
PKC-ε S729E	HA	PKC-ε V5 hydrophobic motif mutant
PKC-ε DM	GFP	PKC-ε ALM + HMM double mutant
PKC-ε TM	GFP	PKC-ε ALM + TMM + HMM triple mutant
PKC-δ/εV5 T505E	GFP	PKC-δ/εV5 activation loop mutant
PKC-ε/δV5 T566E	GFP	PKC-ε/δV5 activation loop mutant
PKC-δ/εV5 DM	GFP	PKC-δ/εV5 ALM + HMM double mutant
PKC-ε/δV5 DM	GFP	PKC-ε/δV5 ALM + HMM double mutant
PKC-δ/εV5 TM	GFP	PKC-δ/εV5 ALM + TMM + HMM triple mutant
PKC-ε/δV5 TM	GFP	PKC-ε/δV5 ALM + TMM + HMM triple mutant
PKC-δ CD24 T505E	GFP	PKC-δ w/ C-terminal deletion of 24 amino acids + ALM
PKC-δ CD54 T505E	GFP	PKC-δ w/ C-terminal deletion of 54 amino acids + ALM
PKC-ε CD6 T566E	HA	PKC-ε w/ C-terminal deletion of 6 amino acids + ALM
PKC-ε CD6 S729E	HA	PKC-ε w/ C-terminal deletion of 6 amino acids + HMM
PKC-ε CD6 DM	HA	PKC-ε w/ C-terminal deletion of 6 amino acids + ALM + HMM

<u>Specific Aims</u>

1. Construction of expression vectors with HA-tagged and GFP-tagged PKC V5 domain chimeric and truncation mutants.

2. Establishment of 32D and NIH 3T3 cell lines that carry the above vectors.

3. Determination of ability of V5 domain to mediate 32D cell differentiation and NIH 3T3 cell transformation.

4. Testing whether the PKC V5 domain modulates kinase activity.

5. Assessing the ability of the V5 domain to alter PKC priming phosphorylation status.

6. Determination whether TPA can restore wild-type phosphorylation status to PKC V5 domain mutants.

7. Assessment of glutamic acid substitution as a priming phosphorylation substitute in novel PKC isoforms.

8. Determination whether PKC V5 chimeras can bind PDK-1.

Wild-Type and Mutant PKC Constructs

Site-Directed Mutagenesis

Plasmids containing PKC-δ, -ε, -δ/εV5 and -ε/δV5 [65] were obtained from Qiming Wang, NCI. PKC-ε/δV5 contained a point mutation in the codon for amino acid 239 (V→A). Site-directed mutagenesis was performed with Quickchange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: forward (F), TTC AAG GAG ATT GAC TGG TCC CTC CTG GAG; reverse (R), CTC CAG GAG GGA CCA GTC AAT CTC CTT GAA.

Site-directed mutagenesis was also employed to change critical priming phosphorylation sites from codons for serine or threonine to one encoding glutamic acid within many of the PKC wild-type, chimeric and truncation fusion proteins. The primers used and the corresponding amino acid substitutions are as follows: PKC-δT505E, (F) GGC CGG GCC AGC GAG TTC TGC GGC ACT, (R) AGT GCC GCA GAA CTC GCT GGC CCG GCC; PKC-εT566E, (F) GGC GTG ACA ACT ACC GAG TTC TGT GGG ACT CCT, (R) AGG AGT CCC ACA GAA CTC GGT AGT TGT CAC GCC; PKC-δS643E, (F) GAA TGA GAA ACC TCA GCT TGA GTT CAG TGA CAA GAA CCT CAT CG, (R) CGA TGA GGT TCT TGT CAC TGA ACT CAA GCT GAG GTT TCT CAT TC; PKC-εT710E, (F) CGC GGG AAG AGC CAA TAC TTG AGC TTG TGG ATG AAG CAA TC, (R) GAT TGC TTC ATC

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CAC AAG CTC AAG TAT TGG CTC TTC CCG CG; PKC-δS662E, (F) GCC TTC

CAT GGC TTC GAA TTT GTG AAT CCC AAG, (R) CTT GGG ATT CAC AAA

TTC GAA GCC ATG GAA GGC; PKC-εS729E, (F) GAA TTT AAA GGC TTC

GAA TAC TTT GGT GAA G, (R) CTT CAC CAA AGT ATT CGA AGC CTT TAA

ATT C; PKC-εCD6S729E, (5') TTA CAT GGT ACC TTG GTA GTG TTC AAT

GGC CTT CTT AAG ATC AAA ATC TGC G, (3') GCC GCG AAT TCA AAA GTA

CTC GAA GCC TTT AAA TTC.

PCR Amplification and Cloning of Wild-Type PKC-δ and -ε as well as Their

Derivative Chimeric and Truncation Mutants

The wild type, chimeric and truncated PKC constructs were subsequently

amplified from pLTR-PKC-δ, -ε, -δ/εV5 or pCRII-PKC-ε/δV5 with 250 ng of plasmid

and 10 pmol of each primer as appropriate by PCR: 10 min, 94°C; 15-24 cycles of 1

min at 94°C, 1 min at 60°C and 3 minutes at 72°C; 10 min at 72°C; hold at 4°C. The

Platinum Pfx PCR Kit (Invitrogen, Carlsbad, CA) with Pfx Polymerase and the

appropriate buffer and nucleotides was used with each reaction. The primers used in

the construction of the HA-tagged PKCs included a KpnI site at the 5' end and an

EcoRI site at the 3' end for ligation. The primers used in the construction of the GFP-

tagged PKC-ε constructs were the same as those for the HA-tagged constructs except

for the 5' primer that included an EcoRI site: TTA CAT GAA TTC TTG GTA GTG

TTC AAT GGC CTT CTT AAG ATC AAA ATC TGC G. Primers were not needed in

the construction of the GFP-tagged PKC-δ constructs, as they could be excised from

the pHM6 vector with HindIII and EcoRI and ligated directly into pEGFP-C2. Primer sequences were as follows: pHM6-PKC-δ, (F) TTA CAT GGT ACC TTG GCA CCC TTC CTG CGC ATC TCC TT, (R) ATA TGA ATT CTT AAA TGT CCA GGA ATT GCT CAA ACT TGG GAT TCA CAA AGG AG; pHM6-PKC-δ/εV5, (F) TTA CAT GGT ACC TTG GCA CCC TTC CTG CGC ATC TCC TT, (R) ATA TGA ATT CAG GGC ATC AGG TCT TCA CCA AAG TAG GA; pHM6-PKC-ε, (F) TTA CAT GGT ACC TTG GTA GTG TTC AAT GGC CTT CTT AAG ATC AAA ATC TGC G, (R) ATA TGA ATT CAG GGC ATC AGG TCT TCA CCA AAG TAG GA; pHM6-PKC-ε/δV5, (F) TTA CAT GGT ACC TTG GTA GTG TTC AAT GGC CTT CTT AAG ATC AAA ATC TGC G, (R) ATA TGA ATT CTT AAA TGT CCA GGA ATT GCT CAA ACT TGG GAT TCA CAA AGG AG; pHM6-PKC-δCD11, (F) TTA CAT GGT ACC TTG GCA CCC TTC CTG CGC ATC TCC TT, (R) ATA TGA ATT CAA AAG GAG AAG CCA TGG AAG GCT TCC TGG; pHM6-PKC-δCD24, (F) TTA CAT GGT ACC TTG GCA CCC TTC CTG CGC ATC TCC TT, (R) ATA TGA ATT CAG ATG AGG TTC TTG TCA CTG AAG GAA AGC TGA G; pHM6-PKC-δCD54, (F) TTA CAT GGT ACC TTG GCA CCC TTC CTG CGC ATC TCC TT, (R) ATA TGA ATT CAG GGC TTA AAG GGC GGC TCC ACC TTC; pHM6-PKC-εCD6, (F) TTA CAT GGT ACC TTG GTA GTG TTC AAT GGC CTT CTT AAG ATC AAA ATC TGC G, (R) ATA TGA ATT CAA AAG TAG GAG AAG CCT TTA AAT TCT TCC TGG TTG ATC TGC; pHM6-PKC-εCD20, (F) TTA CAT GGT ACC TTG GTA GTG TTC AAT GGC CTT CTT AAG ATC AAA ATC TGC G, (R) ATA TGA ATT CAA ATG ATT GCT TCA TCC ACA AGT GTA AGT ATT GGC TCT TCC; pHM6-PKC-εCD50, (F) TTA CAT GGT ACC TTG GTA GTG TTC AAT GGC CTT CTT

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AAG ATC AAA ATC TGC G , (R) ATA TGA ATT CAC GGC TTG AAG GGG
GGC TTG ATT TTC TTC TG. The amplicons were then cloned into the pEGFP-C2
(Clontech, San Diego, CA) and pHM6 (Roche, Indianapolis, IN) mammalian
expression vectors.

Construction of New V5 Chimeras Based on Genomic Structure

The Exon 1-17/1-22 and Exon18/23 fragments of PKC-δ and -ε, respectively,
were obtained using PCR with primers directed to the 5' and 3' ends of the PKC-δ and
-ε fragments. The sequences and locations of the primers were as follows: for the
PKC-δ(Exon1-17) fragment,
TTACATGGTACCTTGGCACCCTTCCTGCGCATCTCCTT (Del5'KpnI) and
CACTTTGGGCTTAAAGGGCGGCTCCAC (δ17R); for the PKC-δ(Exon 18)
fragment, AAATCCCCTTCAGACTACAGCAAC (δ18F) and
ATATGAATTCTTAAATGTCCAGGAATTGCTCAAACTTGGGATTCACAAAGG
AG (Del3'EcoRI); for the PKC-ε(Exon1-22) fragment,
TTACATGGTACCTTGGTAGTGTTCAATGGCCTTCTTAAGATCAAAATCTGCG
(Epi5'KpnI) and AATTCTCGGCTTGAAGGGGGGCTTG (ε22R); for the PKC-
ε(Exon 23) fragment, AAAACCAAAAGAGATGTCAATAACTTTGAC (ε23F) and
ATATGAATTCAGGGCATCAGGTCTTCACCAAAGTAGGA (Epi3'EcoRI)
200 ng of the cloned pHM6-PKC-ε and -δ were used as templates. PCR conditions
were the same as were used for the making of the other constructs. The PKC-δ/ε23

and -ε/δ18 constructs were obtained by blunt end ligation of the δ and ε fragments followed by a second round of PCR amplification using these 5' and 3' primers: pHM6-PKC-δ/ε23, (F) TTA CAT GGT ACC TTG GCA CCC TTC CTG CGC ATC TCC TT, (R) ATA TGA ATT CAG GGC ATC AGG TCT TCA CCA AAG TAG GA; pHM6-PKC-ε/δ18 (F) TTA CAT GGT ACC TTG GTA GTG TTC AAT GGC CTT CTT AAG ATC AAA ATC TGC G, (R) ATA TGA ATT CTT AAA TGT CCA GGA ATT GCT CAA ACT TGG GAT TCA CAA AGG AG; pEGFP-C2-PKC-ε/δ18, (F) TTA CAT GAA TTC TTG GTA GTG TTC AAT GGC CTT CTT AAG ATC AAA ATC TGC G, (R) ATA TGA ATT CTT AAA TGT CCA GGA ATT GCT CAA ACT TGG GAT TCA CAA AGG AG. The pEGFP-C2-PKC-δ/ε23 construct was made by digesting and excising the PKC-δ/ε23 insert from the pHM6 vector with HindIII and EcoRI and re-ligating it into the pEGFP-C2 vector that had been digested with the same restriction enzymes.

Sequence Verification

Sequence verification of all constructs was performed by cycle-sequencing in a Hybaid PCRExpress thermal cycler with 200-500 ng template, 3.2 pmol primer and 8 μl of Terminator Ready Reaction Mix, (Applied Biosystems, Foster City, CA) in a total volume of 20 μl. Reaction cycle was as follows: 1 min at 96°C followed by 25 cycles of 96°C for 30 sec, 50°C for 15 sec, 60°C for 4 min followed by a final cycle at 60°C for one minute. Sequencing reactions were submitted to the NCI Sequencing Mini-Core, and the results were analyzed/verified with Sequencer™ 4.2.2 software.

Cell Culture

32D and WEHI-3 cells were obtained from Linda Wolff, NCI, NIH. NIH 3T3 and Rat-2 cells were provided by Douglas Lowy, NCI, NIH. HEK 293 cells were obtained from Beverly Mock, NCI, NIH. COS-7 and CHO-K1 cells were purchased from American Type Culture Collection (Manassas, VA). All cells were cultured at 37°C with 5% carbon dioxide, 100 U/ml penicillin, 100 μg/mL streptomycin and 200 μM L-glutamine (Biosource, Camarillo, CA). 32D cells were grown in RPMI 1640 medium with 10% fetal calf serum (Hyclone, Logan, UT), 10% WEHI-3-conditioned medium (source of IL-3) and 50 mM β-mercaptoethanol (Sigma, St. Louis, MO). COS-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Calf Serum. NIH 3T3 cells were grown in DMEM with 10% Calf Serum (Invitrogen, Carlsbad, CA). CHO-K1 cells were grown in F-12 Nutrient Mixture (Invitrogen) and 10% fetal calf serum.

Overexpression of PKC Fusion Proteins in 32D, COS-7, NIH 3T3, CHO-K1, RAT-2 and HEK 293 Cells

1×10^7 32D cells in 1 ml of Electroporation Buffer (10 mM NaPO$_4$, 1 mM MgCl$_2$, 250 mM sucrose – pH 7.4) were transfected with 20 μg DNA by electroporation with a BTX Electro Square Porator ECM 830 set at 350 V, for two 99 μs pulses one second apart. These cells were selected for 2 weeks in medium supplemented with 0.5 μg/ml G418 (GIBCO) and then cloned by limiting dilution.

Rat-2, NIH 3T3, CHO-K1, COS-7 and HEK 293 cells were all transiently transfected as appropriate for each cell line according to the Polyfect protocol from Qiagen (Valencia, CA).

Western Blot Analysis

Cells were pelleted and lysed in RIPA buffer (50 mM Tris-pH 7.4, 150 mM NaCl, 1% Triton-X 100, 1% Sodium Deoxycholate, 0.1% SDS) with Protease Inhibitor Cocktail III and Phosphatase Inhibitor Cocktails I and II (Calbiochem, San Diego, CA). 5X Loading Buffer (0.025% SDS, 200 mMTris-HCl-pH 6.8, 50% glycerol 0.2 M dithiothreitol and 0.05% (w/v) bromophenol blue) was added to each sample and samples were boiled at 95°C for 5 minutes and stored at -20°C. Proteins were separated on 4-20% acrylamide gradient tris-glycine Novex gels (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked for 15-20 min with 5% (w/v) nonfat, dried milk in TTBS. Membranes were incubated with primary antibody overnight at 4°C, washed 3 times with TTBS and then incubated with secondary antibody conjugated with horseradinsh peroxidase for 1 hr at room temperature. Immunoreactivity was visualized using SuperSignal ® West Pico chemiluminescence detections system (Pierce, Rockford, IL).

Differentiation Assays

Morphology

32D cells were treated with 100 nM TPA for 8 hours to induce macrophage differentiation. To assess morphology, cytospins of 2-5 x 10^5 TPA-treated and untreated cells were stained with Diff-Quick (American Scientific Products, McGaw Park, IL), and images were collected using an upright bright-field microscope (Zeiss Axiophot, Thornwood, NY) under high magnification. The microscope is fitted with an MTI 3CCD camara and Scion Image 1.62c software (Scion Corp, Frederick, MD).

Flow Cytometry Analysis

1 x 10^6 32D cells, with or without 8-hr treatment with 100 nM TPA, were incubated with anti-Mac1 (CD11b) and anti-Mac-3 (M3/84) antibodies (BD Biosciences, San Diego, CA) conjugated with FITC at 4°C for 30 min. Cells were subsequently analyzed for surface expression of these antigens using a Becton Dickinson FACScan (San Jose, CA). Cells incubated without antibodies or with an irrelevant isoype-matched antibody, FITC anti-mouse CD45R/B220 (BD Biosciences), were used as negative controls.

Confocal Fluorescence Microscopy

Prior to observation, transiently transfected NIH 3T3 cells were washed twice with standard medium (DMEM without phenol red supplemented with 1% FBS) pre-warmed to 37°C. TPA was diluted to 1 μM concentration in the same medium. To image live cells, a Bioptechs Focht Chamber System (FCS2) was inverted and attached to the microscope stage with a custom stage adapter in the confocal minicore (Center for Cancer Research, National Cancer Institute, NIH). NIH 3T3 cells, cultured on a 40-mm round coverslip, were introduced into the chamber system, which was connected to a temperature controller set at 37°C, and medium was perfused through the chamber with a Model P720 microperfusion pump (Instech, Plymouth Meeting, PA). Sequential images of the same cell were collected at 1-min intervals using LaserSharp software through a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a 60X planapochromat lens (Melville, NY). Excitation at 488 nm was provided by a krypton-argon gas laser with a 522/32 emission filter for green fluorescence.

Kinase Assays with Full-Length Substrate

Immunoprecipitated proteins were washed 3 times in kinase buffer (20 mM Tris, pH 7.5, 5 mM $MgCl_2$ and 0.1% NP40), aliquoted into 2 tubes, each containing 20 μl of Protein G-sepharose beads: One tube was used for the kinase assay itself and one

tube for Western blotting. The immunoprecipitates were then resuspended in 20 μl of

reaction buffer with 0.015 mM cold ATP, 0.2 μCi/μl γ^{32}P-ATP (Amersham-Pharmacia,

Piscataway, NJ), 1 μg Histone H1 or Myelin Basic Protein, 1 μM TPA,

phosphatidylserine, and 0.2 mM DTT). Reactions were allowed to proceed for 30 min.

at 30° C, except where otherwise noted. Reactions were stopped by addition of

Western sample buffer and immediately loaded onto polyacrylamide gels. Gels were

stained with Gelcode Blue (Pierce, Rockford, IL), photographed, and then wrapped in

plastic wrap exposed to X-ray film at room temperature for 4-18 hours. Spot

densitometry readings were taken with the Fluorchem™ 8000 system by Alpha

Innotech Corp., San Leandro, CA). Integrated density values for vector alone were

considered background and subtracted from values for experimental samples.

Kinase Assays with Biotinylated Peptide Substrates

Immunoprecipitated proteins were washed three times in kinase buffer (above),

aliquoted into 4 tubes, each containing 10 μl of Protein G-sepharose beads: 3 tubes for

kinase assay replicates and one tube for Western blotting. The immunoprecipitates

were then resuspended in 20 μl of reaction buffer recommended by the Calbiochem

PKC Assay Kit: 0.015 mM cold ATP, 0.2 μCi/μl γ^{32}P-ATP (Amersham-Pharmacia,

Piscataway, NJ), 0.025 mM biotinylated PKC pseudosubstrate, 0.5 mM CaCl$_2$, 10 mM

MgCl$_2$, 20 mM Tris-HCl pH 7.5, and an activator micell suspension solution composed

of 0.3 mg/ml PS, 0.03 mg/ml DAG and 0.3% Triton X-100. Reactions were allowed to

proceed for 30 min. at 30° C, unless otherwise noted, and were stopped by the addition

of 10 μl of 8.0 M guanidine hydrochloride. Labeled pseudosubstrate was bound to a membrane within a centrifugal ultrafiltration unit that was washed and added directly to scintillation fluid for counting.

Immunoprecipitation

All PKC constructs and GFP were immunoprecipitated from HEK or CHO cell lysates in the presence of 2 μl anti-GFP (BD Biosciences), 40 μl Protein G-sepharose and 750 μl RIPA buffer with Protease Inhibitor Cocktail III (Calbiochem) and Phosphatase Inhibitor Cocktails I + II (Calbiochem). Sepharose beads were washed three times with 400 μl of kinase buffer (5 mM $MgCl_2$, 1% NP40 and 20 mM Tris, pH 7.5). Immunoprecipitated proteins were separated by SDS-PAGE and transferred to Protran nitrocellulose membranes (Schleicher & Schuell).

Co-immunoprecipitation

PDK-1 was immunoprecipitated with 30 μl of protein G-sepharose beads, 2 μl rabbit anti-PDK-1 Ab (#3062, Cell Signaling) from HEK cells lysed with 20 mM Tris (pH 7.9), 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 10% glycerol, 1% Triton-X 100 and Protease Inhibitor Cocktail III (Calbiochem, San Diego, CA). The above contents were tumbled overnight at 4° C. Immunoprecipitation complexes were then washed 1X in lysis buffer, 1X in high salt buffer (20 mM HEPES, 500 mM NaCl,

0.1% Triton-X 100, 10% glycerol) and finally 2X in low salt buffer (20 mM HEPES,

150 mM NaCl, 0.1% Triton-X 100 and 10% glycerol).

THE ROLE OF THE V5 DOMAIN IN CONFERRING ISOFORM-SPECIFIC FUNCTION

Expression of Wild-Type, Chimeric and Truncated PKCs in 32D Cells

In cell differentiation and cell transformation assays, PKC-ε and -δ isoform-specific function was previously mapped to the C-terminal half those isoforms [45, 46]. Those experiments have been summarized in the Introduction. In the following experiments, PKC-δ and -ε V5 chimeras and truncation mutants were designed, constructed and tested within the context of 32D cell differentiation assays to see if isoform-specificity could be mapped more precisely to the most carboxyl-terminal (V5) domain of these nPKC isoforms.

PCR-generated PKC-δ, -ε, -δ/εV5, -ε/δV5 as well as PKC-δ and -ε C-terminal truncation mutants were first subcloned into the pHM6 mammalian expression vector for generation of HA epitope-tagged proteins. Figure 3 depicts the V5 sequences for the chimeric and truncation mutants utilized throughout this study. The truncation mutants were designed to include either both, one or none of the phosphorylation priming sites located within the V5 domain. The entire V5 domain has been deleted in the most truncated PKC-δ and -ε mutants. The sequences exchanged between the chimeras include all of the V5 domain as well as a short sequence of approximately 15 amino acids just upstream with > 81% homology/62% identity at the amino acid level.

To determine the role of the V5 domain in conferring the isoform-specific ability of PKC-δ to induce 32D promyeloid cells to differentiate into macrophage cells,
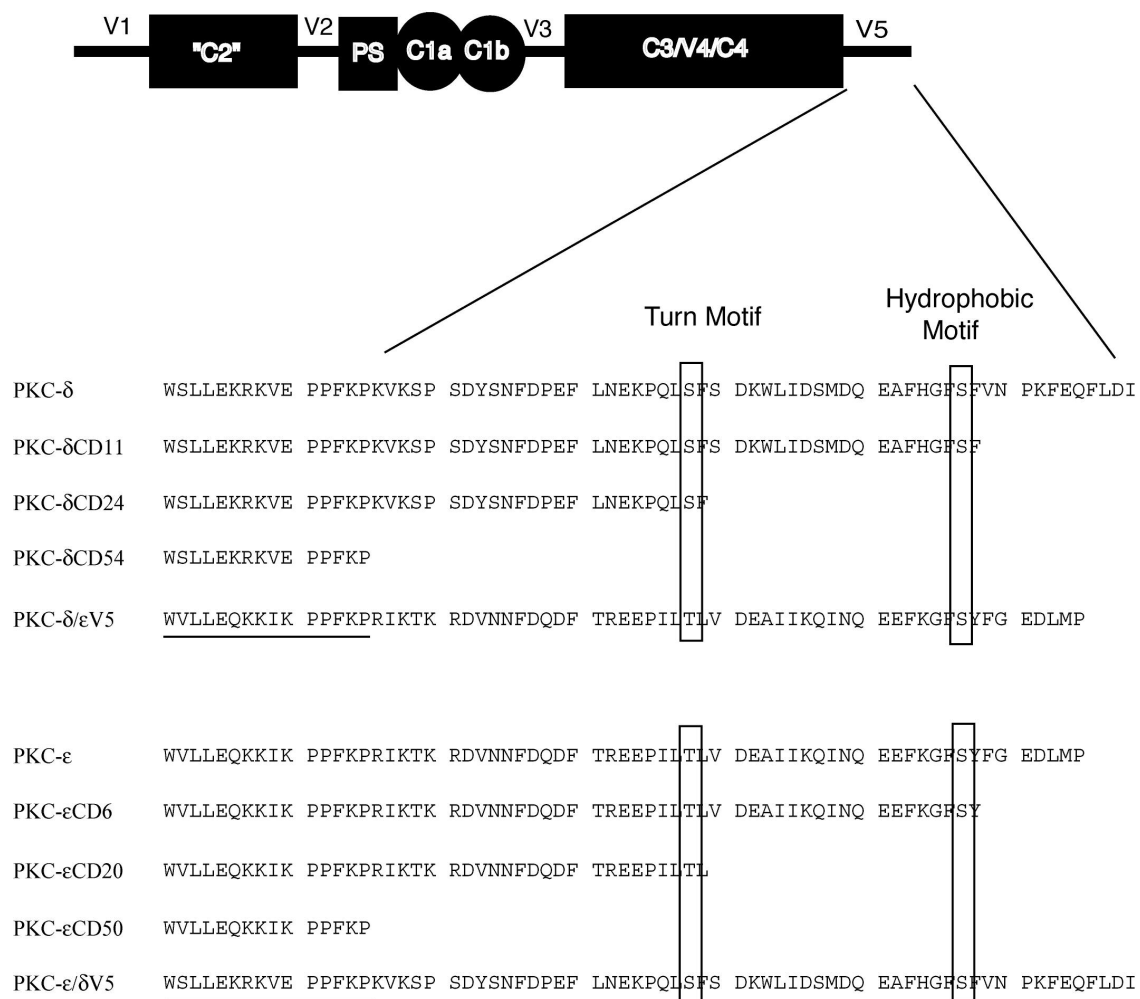
Figure 3. V5 sequence of wild-type murine PKC-δ and -ε and their derivative mutants. Turn motif and hydrophobic motif phosphorylation sites are boxed. Underlined sequence just upstream of the V5 domain was included in the chimeric exchange as a structural link between the two variable domains. "CD" followed by a number indicates a C-terminal deletion of the indicated number of amino acids.

32D cell clones expressing truncated and chimeric PKC mutants were selected and tested. Because levels of protein expression may affect protein function in cells, we sought lines of 32D cells that stably expressed relatively equal amounts of HA-PKC fusion protein. Figure 4 shows the results of a representative Western blot of PKC-expressing clones. This blot is a compilation of those clones with the most comparable levels of PKC expression as determined by staining with anti-HA, anti-PKC-δ and anti-PKC-ϵ antibodies. 32D cells overexpressing PKC-δ, -ϵ and -δ/ϵV5 were readily obtained. However, 32D cell clones overexpressing comparable amounts of PKC-ϵ/δV5 and PKC truncation mutants were much more difficult to find, even after intensive screening of at least 25 clones per construct. No endogenous PKC-ϵ was found in 32D cells, but a small amount of endogenous PKC-δ could be seen with the anti-PKC-δ antibody.

32D Cell Differentiation

32D cells are bi-potential promyelocytic cells capable of differentiating into either granulocytes or macrophages depending upon the conditions. 32D cells that overexpress PKC-δ, but not those that overexpress PKC-ϵ, differentiate into macrophages in the presence of TPA [36]. Mischak *et al.* showed that overexpressed wild-type PKC-δ in 32D cells could induce macrophage differentiation upon stimulation with as little as 16 nM TPA for anywhere from 4-18 hours [36]. In this system differentiation starts out with adhesion of greater than 90% of the 32D cells to the bottom of the culture dish. After several hours of TPA stimulation, most cells have
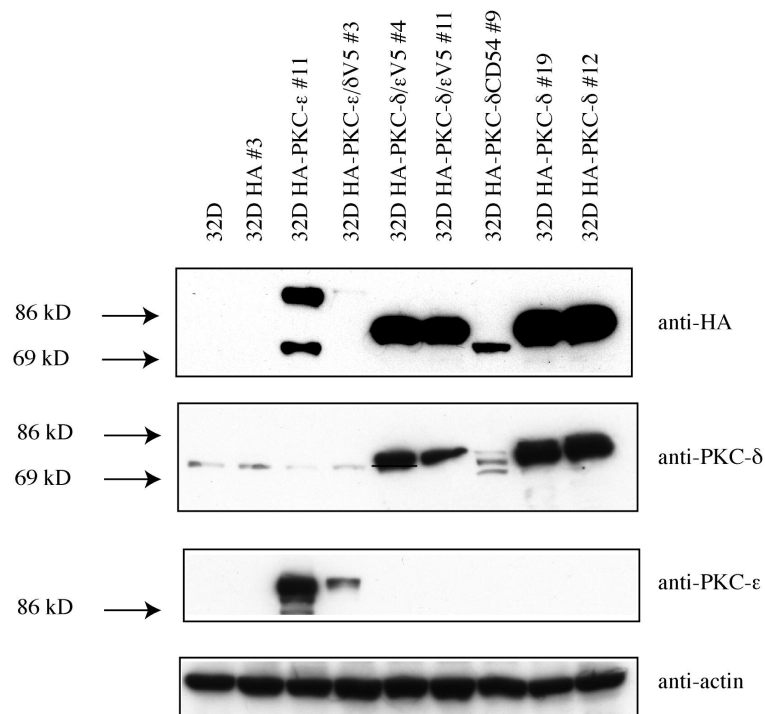
Figure 4. Western blot determination of mutant PKC expression in 32D cell clones. Overexpressed proteins, indicated at the top of each lane, were stained with either anti-HA or an anti-N-terminal Generally, 15 μg of total protein lysate was loaded in each lane. PKC-δ or anti-PKC-ε antibody. Staining for actin shows that equal amounts of total protein were loaded in each lane. Positions of protein size standards are shown to the left.

returned to their suspended state, but a large fraction remain adherent and have upregulated macrophage-specific surface markers as described in the Introduction. In addition, these cells have increased lysozyme production and are capable of ingesting yeast cells [36].

Wang *et al.* later showed that this PKC-δ-specific ability was conferred to a PKC-εδ chimera that has a PKC-ε regulatory domain and a PKC-δ catalytic domain [46]. With the knowledge that this isoform-specific ability was localized to the catalytic domain, we decided to see if this PKC-δ-specific function could be further mapped to the V5 domain. As a starting point, 32D cell lines overexpressing wild-type PKC-δ and -ε as well as the PKC-δ and -ε V5 chimeras were stimulated with 100 nM TPA for 7 hours to see if either chimera would mediate 32D cell differentiation.

Histological analysis of those lines tested showed that only PKC-δ-overexpressing 32D cells acquired macrophage-like phenotypic features: large cells with complex plasma membrane structures and numerous cytoplasmic vacuoles (Figure 5, Panel D). Interestingly, PKC-δ overexpressing 32D PKC-δ cells are less polarized than cells overexpressing other PKCs before TPA stimulation (Figure 5, Panels A,C,E, G, and H). 32D PKC-δ/εV5 cells seem somewhat enlarged after TPA treatment (Figure 5, Panel H) and a small portion of 32D PKC-ε/δV5 cells have extended pseudopodia, as have 32D PKC-δ cells (Figure 5, Panels D and J). Although all 32D cells showed an increase in adherence to the bottom of the cell culture dish shortly after TPA stimulation, only PKC-δ-overexpressing cells continued to adhere to the bottom of the cell culture dish after 7 hours of TPA stimulation (data not shown). These

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No TPA, 7 hr   100 nM TPA, 7 hr

Empty vector

PKC-δ

PKC-ε

PKC-δ/εV5

PKC-ε/δV5
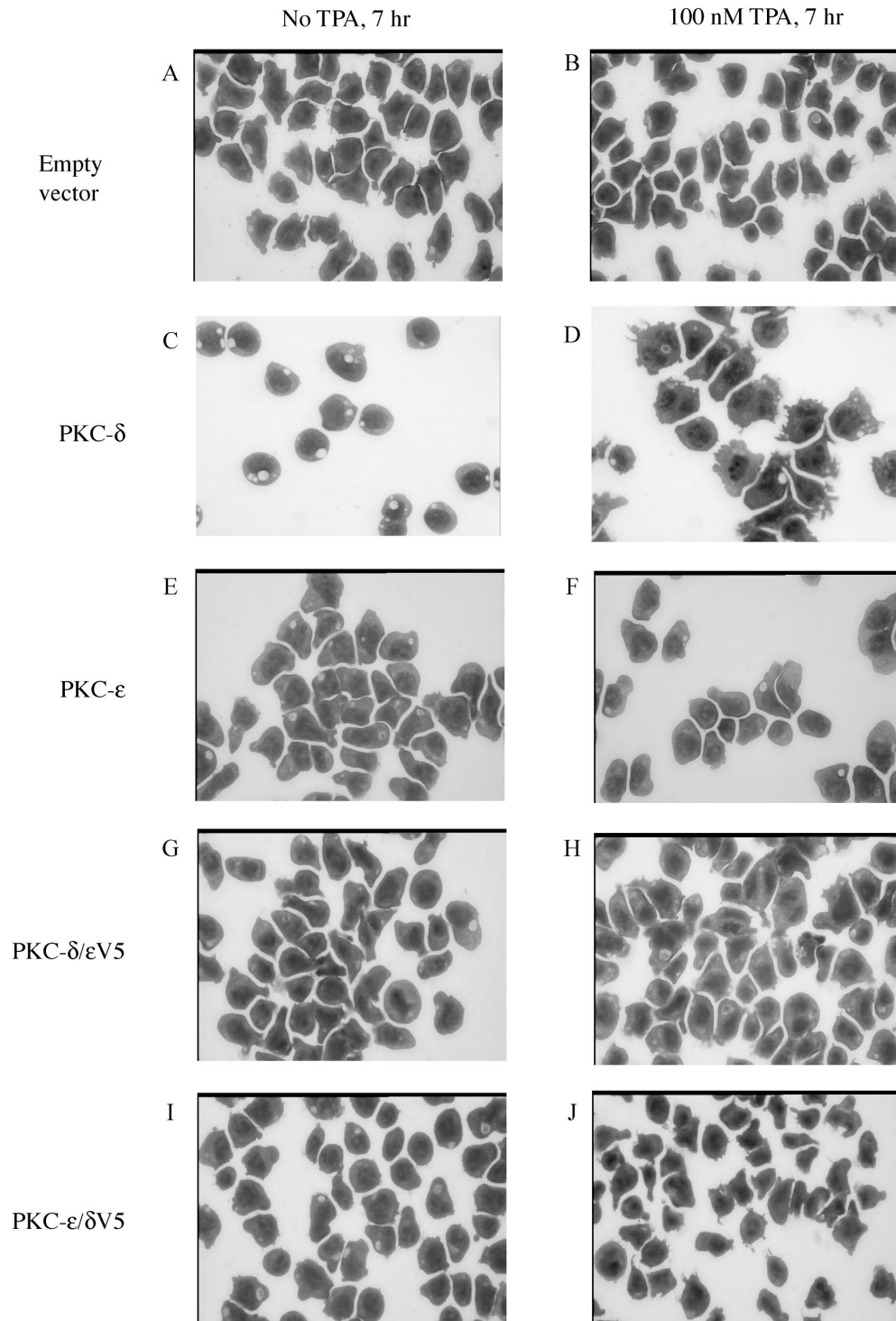
Figure 5. Morphology of wild-type and chimeric PKC overexpressers after stimulation with TPA. 32D Lines that express PKC-δ, -ε, δ/εV5 and -ε/δV5 were treated with 100 nM TPA for 7 h. The cells were applied to uncoated glass microscope slides using cytocentrifugation and then stained with Giemsa. Photographs of representative fields were taken using a light microcope with a 40X objective. Data are from one of three similar experiments, all of which showed clear evidence of macrophage morphology only in the TPA-treated wild-type PKC-δ overexpressing cells.

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adherent cells showed a flattened, polarized morphology, similar to that seen in Figure 5, Panel D. In summary, the morphology data indicate that PKC-δ-specific 32D cell differentiation could not be attributed to PKC-δ's V5 domain, since overexpression of either chimera induced certain morphological features indicative of a partial and unconvincing macrophage differentiation phenotype.

FACS analysis for surface markers characteristic of macrophages also indicated that the V5 domain within the context of PKC-ε and -δ V5 chimeras was unable to mediate 32D cell differentiation (Figure 6). While PKC-δ-overexpressing 32D cells showed an increase in Mac-1 and Mac-3 expression after stimulation with TPA, PKC-ε-, -ε/δV5- and -δ/εV5-overexpressing cells did not (Figure 6). Both sets of results suggested that the V5 domain of PKC-δ was indeed important for conferring isoform-specific function since a PKC-δ molecule with an altered V5 domain was unable to induce macrophage differentiation. However, the PKC-δ V5 domain was not sufficient to confer the isoform-specific function to PKC-ε within the context of a PKC-ε/δV5 chimera. Longer treatments with higher doses of TPA were also unable to induce macrophage differentiation in any but the PKC-δ-overexpressing 32D cell lines (data not shown).

It has already been shown that 32D cell differentiation occurs only in the presence of *overexpressed* PKC-δ [36]. To ensure that 32D cell differentiation was not, however, dependent upon the level of PKC-δ overexpression, 32D cells were transfected cells with PKC-δ under the control of three different promoters: a metallothionine (MTH) and a long terminal repeat (LTR) promoter, in addition to the
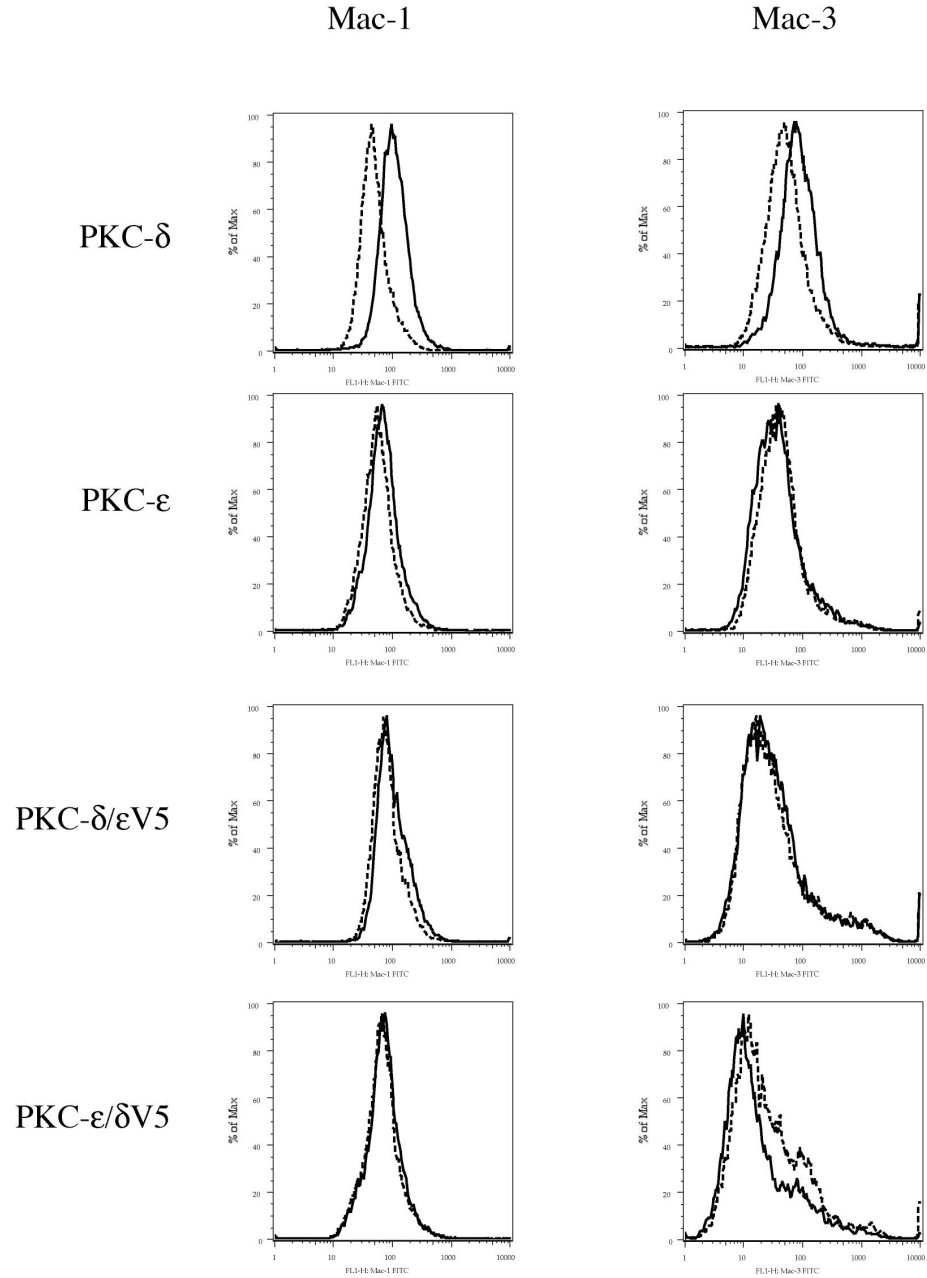
Figure 6. Flow cytometry analysis of 32D cells overexpressing wild-type and V5 chimeric PKCs.
Cells were treated with (solid lines) or without (dashed lines) 16 nM TPA for 10 h and stained with
FITC-labeled antibodies against Mac-1 and Mac-3.

cytomegalovirus (CMV) promoter present within the pHM6 plasmid. This was an important control, inasmuch as some of the mutants and chimeric constructs achieved variable levels of exogenous PKC expression. The LTR promoter-driven PKC-δ plasmid generated the highest level of PKC-δ protein and the metallothionine promoter the least (Figure 7). The resulting cells with varying levels of PKC-δ overexpression were then assayed for differentiation. All three cell lines were capable of differentiating upon TPA stimulation as evidenced by expression of Mac-3 (Figure 7) and as evidenced by histology (data not shown). The results of this experiment suggest that even moderate levels of PKC V5 chimera expression would be enough to induce macrophage differentiation in 32D cells if the chimera were capable of doing so.

32D Cell Proliferation Rates

Wang, *et al.* previously reported that PKC-δ-overexpressing 32D cells showed a slower rate of proliferation than untransfected 32D cells or 32D cells overexpressing PKC-ε [46]. In the same report, this isoform-specific phenomenon was shown to be conferred by the catalytic half of the PKC-δ molecule in the context of PKC-ε and -δ regulatory/catalytic domain chimeras. To see if this isoform-specific function could be conferred by the V5 domain, proliferation rates were recorded for 32D cells overexpressing wild-type as well as PKC-δ and -ε V5 chimeras. Measured in duplicate, the results suggest that there is no difference in proliferation rate between 32D cells expressing wild-type vs. mutant PKC constructs (Table 2). The earlier
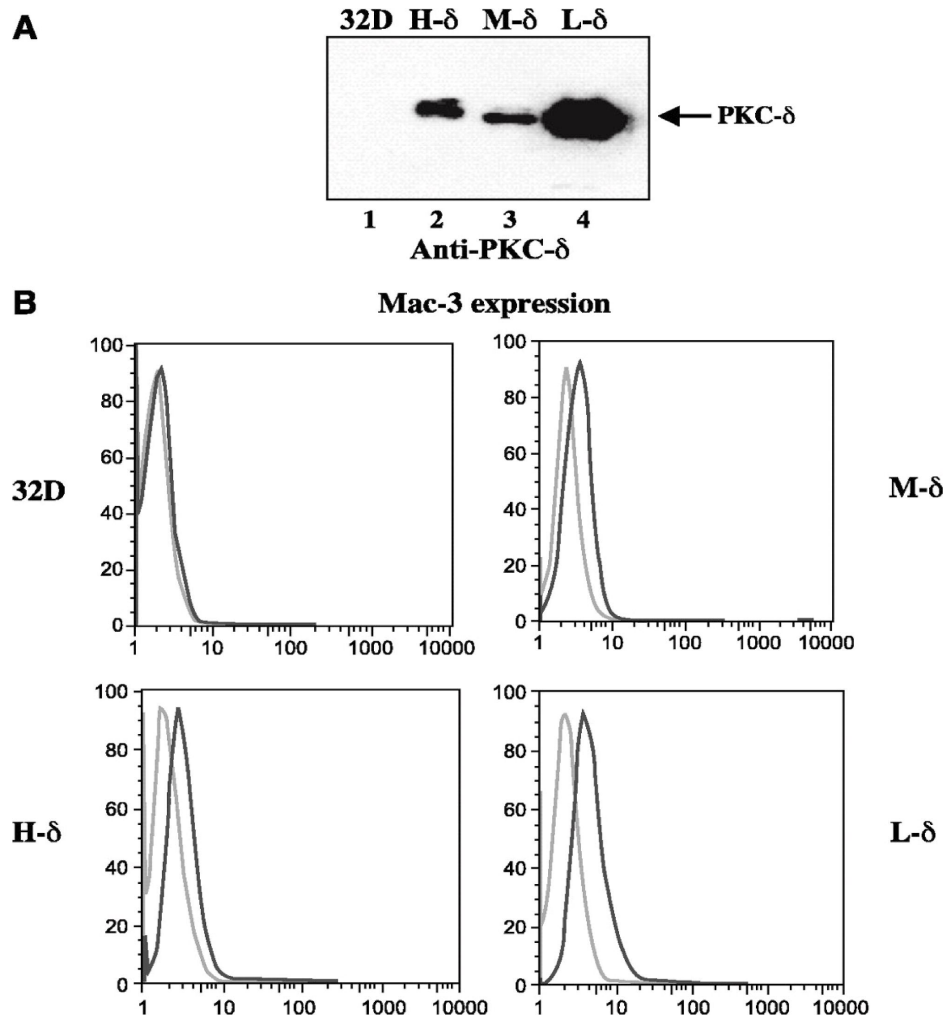
Figure 7. Mac-3 stains positive on TPA-treated PKC-δ-overexpressing 32D cells indicating macrophage differentiation occurs regardless of levels of PKC-δ expression A. Western blot of 32D cell lysates obtained before TPA treatment and developed with anit-PKC-δ. *Lane 1*, untransfected 32D cell lysate. *Lanes 2, 3 and 4*, 32D cells overexpressing varying amounts of PKC-δ protein. *Lane 2*, PKC-δ expressed from the pHM6 vector (H-δ) has a slightly higher molecular weight because this protein is fused to an amino terminal hemaglutinin tag. *Lane 3*, PKC-δ expression with a metallothionine promoter (M-δ). *Lane 4*, PKC-δ expression with a long terminal repeat promoter (L-δ). B. Flow cytometric analysis of the same cells before (gray) and after (black) 6 h treatment with 50 nM TPA.

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Table 2. Doubling time of 32D cells overexpressing wild-type and V5 chimeric PKCs. Results in bold are the average of two experiments (individual results shown in parentheses).

Cell Line	Doubling Time (h)
32D HA vector	**12.5** (11.5, Expt. 1; 13.5, Expt. 2)
32D HA-PKC-δ	**13.6** (13.3, Expt. 1; 13.9, Expt. 2)
32D HA-PKC-ε	**13.8** (15.8, Expt. 1; 11.8, Expt. 2)
32D HA-PKC-δ/εV5	**13.4** (16.4, Expt. 1; 10.4, Expt. 2)
32D HA-PKC-ε/δV5	**10.4** (10.9, Expt. 1; 9.8, Expt. 2)

experiments were performed with PKC-δ and -ε clones that expressed extremely high levels of exogenous PKC. It is likely that the level of expression achieved with the pHM6 vector was not high enough to induce phenotypic changes without the addition of TPA stimulation.

Summary of the V5 Domain's Ability to Confer Isoform-Specific Function

The V5 domain was shown to be essential for PKC function: the exchange of the V5 domain abrogated PKC-δ's ability to mediate macrophage differentiation. However, the PKC-δ and -ε V5 chimeras were unable to confer isoform-specific function in the 32D differentiation assay. Similarly, exogenously expressed PKC-δ truncation mutants did not induce 32D cells to differentiate upon TPA stimulation. PKC-δ truncation mutants and the PKC-ε/δV5 chimera were difficult to express at levels similar to wild-type PKC-δ within this cell line. Thus, even small deletions within the V5 domain or the exchange of the V5 domain among novel PKC isoforms were enough to cause large differences in PKC regulation and function. Further experiments were needed to elucidate the mechanism(s) responsible for those differences.

THE V5 DOMAIN OF PROTEIN KINASE C-δ AND -ε MODULATES KINASE ACTIVITY

PKC Translocation

One possible explanation for the inability of the PKC-δ and -ε V5 chimeras to confer isoform-specific function could be a lack of kinase activity in the unnatural chimeric proteins. This seemed unlikely since chimeric PKCs have been used with success before (see Introduction). Nevertheless, even slightly reduced kinase activity in the V5 chimeras might explain the lack of conferral of isoform-specific function already observed (Chapter 3). We therefore chose to determine the kinase activity of the chimeric and truncated PKCs relative to wild-type PKC-δ and -ε.

PKC is thought to be active only in response to changes in second messenger concentrations (or phorbol esters) which enable the enzyme to translocate - with help from PKC binding proteins - from its resting state in the cytosol to the plasma membrane or other known targets. With this in mind, PKC translocation studies were chosen as a starting point for determining PKC mutant activity. Figure 8 shows the subcellular localization of exogenous PKC constructs in the absence of TPA, as well as the TPA-induced translocation pattern of GFP-tagged PKC-δ, -ε, -δ/εV5 and -ε/δV5. All constructs were present in the cytosol before TPA stimulation; GFP-PKC-δ and -ε/δV5 showed increased nuclear fluorescence due to the nuclear localization signal present within the PKC-δ V5 domain. After TPA addition, PKC-δ rapidly translocated to the plasma and nuclear membranes, whereas PKC-ε translocated only to the plasma

1 μM TPA

| | 0 min | 10 min | 20 min |

GFP-PKC-δ

GFP-PKC-ε

GFP-PKC-ε/δV5

GFP-PKC-δ/εV5
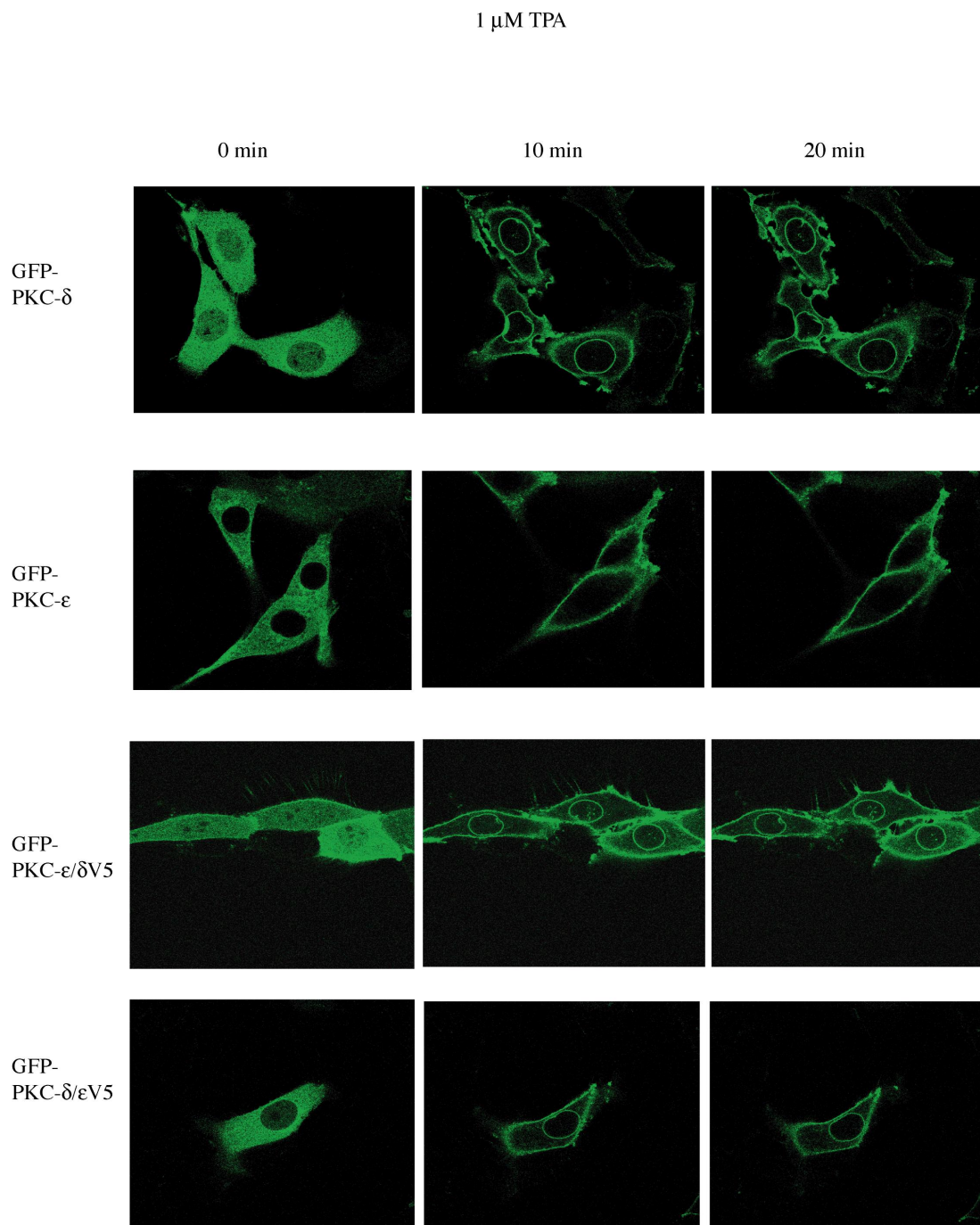
Figure 8. Translocation of GFP-tagged wild-type and chimeric PKCs in response to TPA in NIH 3T3 cells. Cells were imaged every minute for a total of 20 min following the introduction of 1 μM TPA with images indicated times. Single-slice confocal images were captured using a 60X objective and uv illumination.

membrane. Both PKC-δ/εV5 and -ε/δV5 were capable of translocation and responded to TPA stimulation in a manner similar to PKC-δ, i.e. translocation to both the plasma and nuclear membranes. The ability of PKC-δ/εV5 and PKC-ε/δV5 to translocate in response to TPA stimulation suggested that these chimeras were receptive to TPA stimulation and could indeed find their subcellular targets and function as active kinases.

That the translocation pattern of both V5 chimeras resembles that of PKC-δ indicates that the V5 domain is involved in the translocation pattern differences between PKC-δ and -ε. The binding domain of the PKC-δ RACK, p32, is not known, but if other RACK binding domains are an indication, both N-terminal and C-terminal residues are involved [66]. It would be easy to attribute PKC-δ translocation to the nuclear envelope as a consequence of PKC-δ-specific localization within the nucleus, but fluorescent intensity measurements indicate that there is little difference between the amount of PKC-δ and -ε within the nucleus (data not shown) although it does look like there is increased nuclear fluorescence in PKC-δ and PKC-ε/δV5 in Figure 8.

Kinase Assays

In vitro kinase assays were then performed with immunoprecipitated PKCs to see how well PKC chimeric and truncation mutants were able to phosphorylate both full-length and peptide substrates. The kinase assays were performed with γ^{32}P-labeled ATP to measure phosphotransfer to the substrate. This was done by separating the contents of the kinase reaction via SDS PAGE and then exposing the gel to X-ray film.

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The amount of ^{32}P in the target protein band, identified by protein stain, represented the kinase activity in the lysate. Figure 9 shows the results of representative kinase assay performed with wild-type PKC-δ and PKC-δ mutants. Only wild-type PKC-δ was able to phosphorylate full length Histone H1 efficiently. PKC-δ truncation of just 11 amino acids or more from the carboxyl terminus significantly reduced kinase activity, as did the exchange of the PKC-ε V5 domain for the PKC-δ V5 domain (Figure 9). For each kinase assay Western blots and Coomassie staining of the gels were done in parallel to provide controls for both the amount of enzyme used (Western blot) as well as the amount of substrate included in the reaction (Coomassie stained gel). In certain cases densitometry was also utilized as another means of visualizing substrate phosphorylation levels. The densitometry in Figure 9 shows that there was indeed some phosphorylation above background from both the truncated as well as the chimeric PKC-δ mutants.

Figure 10 shows that a C-terminal deletion mutant of six amino acids from PKC-ε allows it to retain significant kinase activity against full length Histone H1, but a C-terminal truncation of 20 amino acids abrogates kinase activity in PKC-ε. As with its reciprocal partner, the kinase activity of the HA-tagged PKC-ε/δV5 is also severely reduced. Interestingly, wild type PKC-ε consistently showed lower kinase activity than PKC-δ; not coincidentally, the level of PKC-ε overexpression was usually below that of wild-type PKC-δ (see Figure 11 and Figure 13).

It is important to note that Coomassie stains of Histone H1 always show a doublet because the purification of Calf Thymus Histone H1 by Calbiochem yields two isoforms [67]. Changes in electrophoretic mobility of these isoforms were not
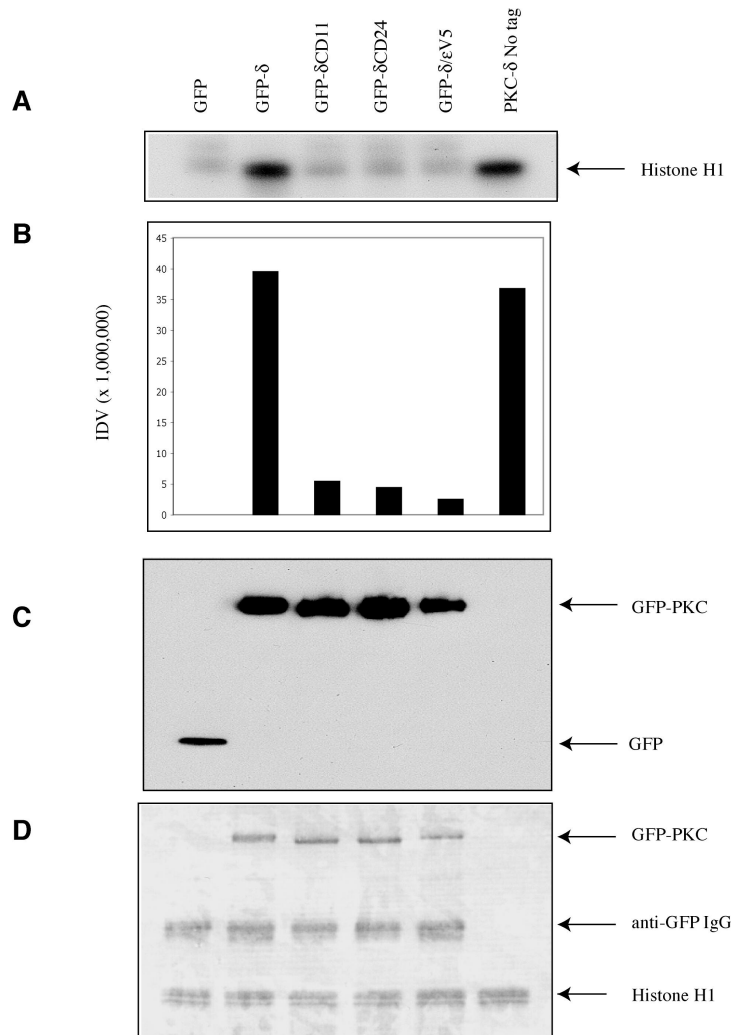
Figure 9. Kinase assay of wild-type PKC-δ and truncation and chimeric mutant derivatives of PKC-δ. GFP-tagged wild-type and chimeric PKC-δ and two C-terminal truncation mutants missing either the last 11 (GFP-δCD11) or 24 (GFP-δCD24) amino acids were immunoprecipitated from transiently transfected COS-7 cells with anti-GFP antibody and Protein A/G agaraose beads. The bead-bound enzyme was then allowed to phosphorylate Histone H1, a known PKC substrate, in the presence of γ^{32}P-ATP, TPA, Mg^{2+}, PS and cold ATP for 30 min at 30°C. The reaction contents were then run on two SDS-PAGE gels in parallel. One gel was Coomassie-stained and then exposed to film. The other was used for a Western blot. Recombinant PKC-δ (0.1 μg) was used as a positive control and included in the last lane. A. Autoradiograph of gel exposed to film. B. Densitometric analysis of the autoradiograph in A. Value of GFP alone was considered background and substracted. (IDV, integrated densitometry values). C. Western blot of the kinase reaction contents stained with anti-GFP shows that similar amounts of PKC-δ were used in each assay. D. Coomassie stain of the gel shows that equal amounts of Histone H1 were used. Note that Calf Thymus Histone H1 yields two isoforms that run as a doublet, both bands of which were phosphorylated.
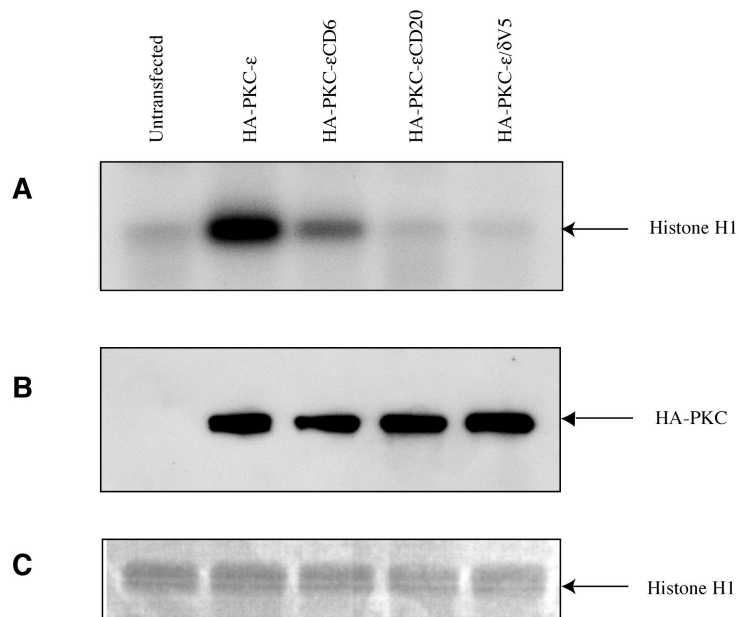
Figure 10. Kinase assay of PKC-ε, PKC-ε/δV5 and PKC-ε truncation mutants. HA-tagged PKC-ε, -ε/δV5 and two PKC-ε C-terminal truncation mutants of 6 and 20 amino acids (PKC-εCD6 and PKC-εCD20) were immunoprecipitated from transiently transfected CHO cells with anti-PKC-ε antibody and Protein A/G agarose beads. The bead-bound enzyme was then allowed to phosphorylate Histone H1, and the kinase assay was completed under conditions identical to those previously described. A. Autoradiograph showing ^{32}P-labelled Histone H1. B. Western blot of immunoprecipitated proteins stained with anti-HA antibody shows that similar amounts of HA-PKC were used in the assay. C. Coomassie stain of polyacrylamide gel shows that equal amounts of Histone H1 were used in the assay.

observed even when strong kinase activity was apparent by autoradiogaphy. This may

be due in part to the 4-20 % polyacrylamide gel gradient, which may have masked real

changes in mobility from phosphorylation. In addition, phosphorylation was likely

distributed between both isoforms, preventing detection of a change in mobility in any

one specific band.

To show that the phosphorylation of Histone H1 was PKC-specific and not the

result of a contaminating kinase present either within the precipitated immunocomplex

or the purified Histone H1 preparation, a PKC inhibitor was used to abrogate kinase

activity. As can be seen in Figure 11, GF109203X, an inhibitor of PKC-α, -βI-II, -γ, -δ

and -ϵ, was able to prevent phosphorylation of Histone H1 by wild-type PKC-δ very

efficiently.

So far, all constructs tested were tagged at the amino terminal portion of the

protein. Because it was possible that an N-terminal tag was inhibiting kinase activity

of the enzymes, we tested the activity of two wild-type PKC-δ fusions proteins, one

with a GFP tag at the N terminus and the other with a GFP tag at the protein's C

terminus. As can be seen in Figure 12, both versions of wild-type PKC-δ were active.

Thus it appears that the location of the tag was not a factor in PKC activity in this

assay. This is evident not only by the ability of both tagged versions of PKC-δ to

phosphorylate Histone H1, but also by their ability to self-label with γ^{32}P at their

autophosphorylation site (Figure 12). The PKC-δ/ϵV5 chimera and two truncated

versions of PKC-δ were unable to phosphorylate Histone H1 as observed in previous

assays. In confirmation of this result, none of these constructs were able to

autophosphorylate at the turn motif (Figure 12). In the case of the PKC-δ/ϵV5
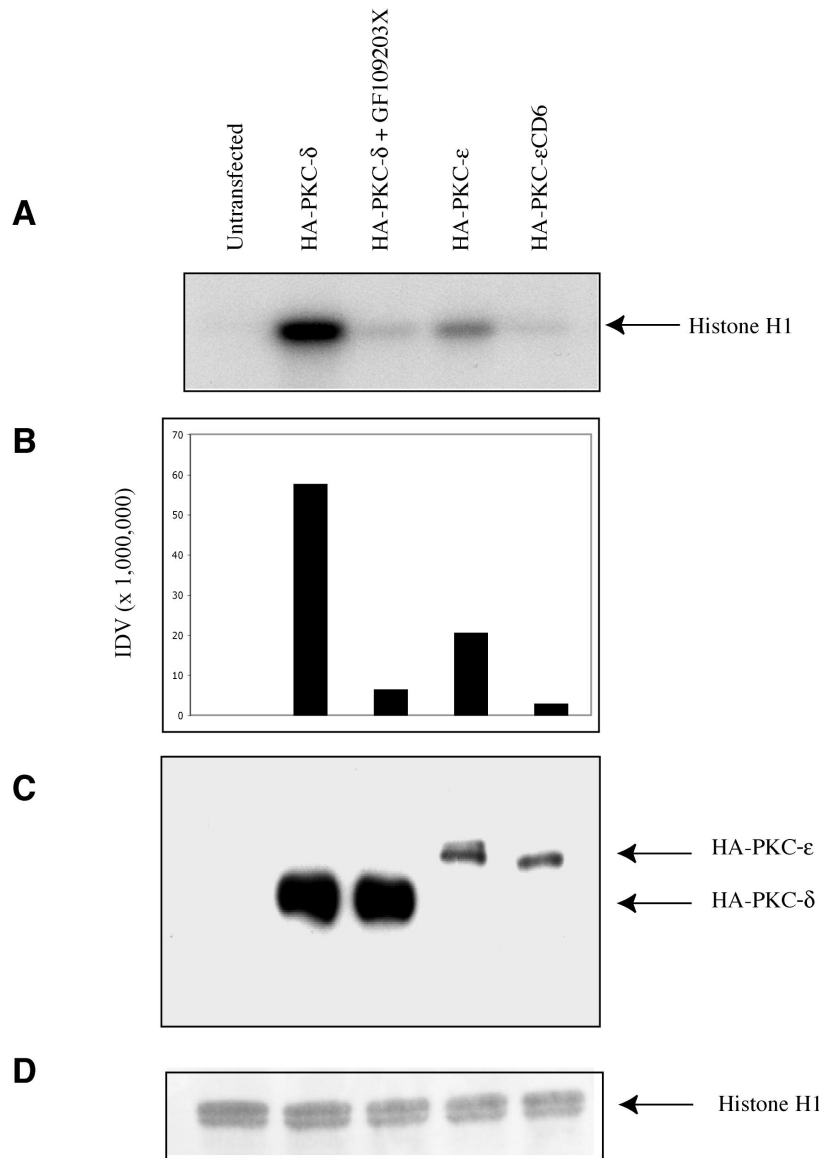
Figure 11. Kinase assay of wild-type PKCs and the effect of a PKC-specific inhibitor. HA-tagged PKC-δ, -ε and -εCD6 were immunoprecipitated from transiently transfected CHO cells with anti-HA antibody and Protein A/G agarose beads. The bead-bound enzyme was then allowed to react with Histone H1 for 15 min at 30°C in the same reaction buffer as previously used with or without 1 μM GF109203X as indicated. A. Autoradiograph showing ^{32}P-labelled Histone H1. B. Densitometric analysis of the autoradiograph in A (IDV, integrated densitometry values). C. Western blot of immunoprecipitated proteins stained with anti-HA antibody. D. Coomassie stain of polyacrylamide gel shows that equal amounts of Histone H1 were used in each reaction.
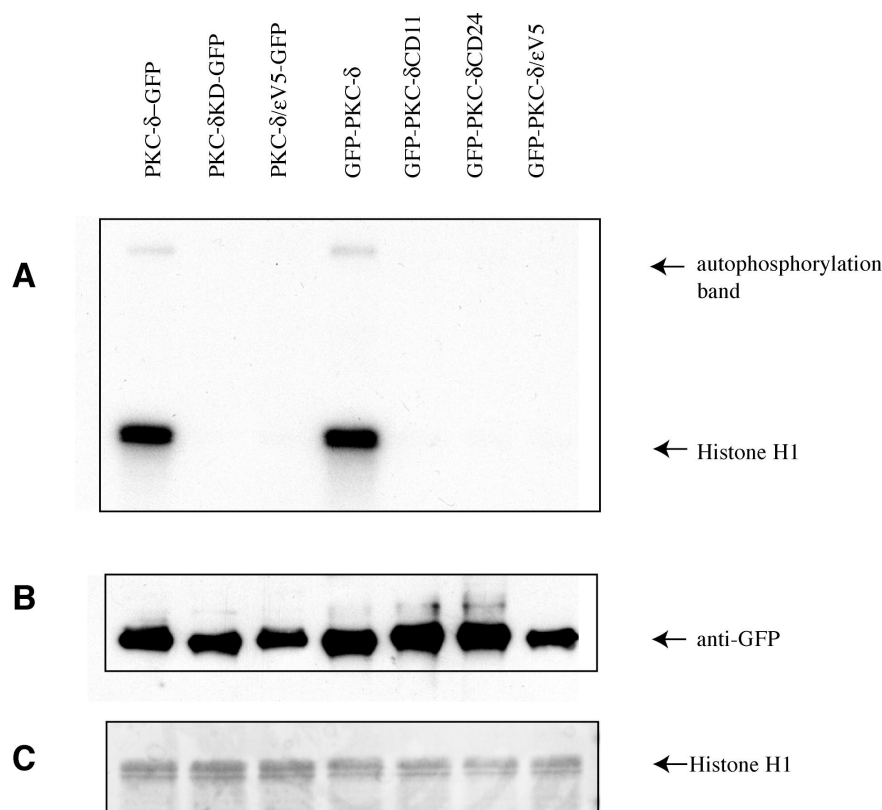
Figure 12. Kinase assay with the GFP tag either N-terminal or C-terminal of the inserted PKC. Immunoprecipitated PKC-δ and PKC-δ/εV5 with GFP tags fused either N-terminal or C-terminal were allowed to phosphorylate Histone H1 for 30 min at 30°C under in the same buffer and conditions previously described. In addition, two N-terminally-tagged PKC-δ C-terminal truncation mutants were included as well as a C-terminally-tagged kinase-deficient PKC-δ construct as a negative control. A. Autoradiograph showing ^{32}P-labelled Histone H1 as well as autophophorylated PKC-δ. B. Western blot of immunoprecipitated proteins stained with anti-GFP antibody shows that similar amounts of GFP-PKC were used in each assay. C. Coomassie stain of polyacrylamide gel shows that equal amounts of Histone H1 were used.

chimera, both an N-terminal GFP tag and a C-terminal GFP fusion protein were used, and neither was able to phosphorylate Histone H1 *in vitro* (Figure 12).

A known kinase-deficient PKC-δ construct was also utilized in this experiment. This version of PKC-δ is mutated at the ATP-binding site (K378R), preventing the enzyme from binding to ATP and, consequently, preventing phosphotransfer [68]. Lane 2 of Figure 12 shows that this construct was unable to phosphorylate Histone H1 or itself and served as a negative control for kinase activity.

The *in vitro* kinase assays performed up to this point clearly showed that wild-type PKC-δ and -ε were catalytically active against Histone H1, yet the kinase assay results for the chimeras were more difficult to interpret. Densitometry and autoradiography results sometimes revealed PKC mutant activity above background (Figure 9) and sometimes they did not (Figure 12). Yet based on the ability of the V5 chimeras' ability to translocate (Figure 8) and based on published success with PKC-α/βII chimeras (see Introduction), it seemed unlikely that the PKC-ε/δV5 and -δ/εV5 chimeras were *completely* inactive. Translocation of PKC from the cytosol to the membrane is often used to define PKC activity because the membrane interaction provides the energy to release the pseudosubstrate domain, allowing the catalytic half of the molecule to interact with and phosphorylation substrate [23]. Moreover, it often appeared that some V5 chimeric kinase activity could be seen, but a large degree of background signal on the autoradiograph in lanes with the negative control complicated the analysis of the level of phosphorylation observed (data not shown). Problems with high levels of background were often a complication when full length Myelin Basic Protein was used as a PKC substrate in the reaction (data not shown). To address these

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issues, experiments were proposed to test the mutants' activity in an altered format and against other PKC substrates, including peptide targets that might pose less of a challenge to mutant PKC proteins, in terms of binding and/or specificity.

A slightly varied kinase assay protocol was adopted which utilized a Protein Kinase C Assay Kit from Calbiochem (San Diego). This new protocol presented a number of advantages. First, the assay was slightly quicker. The wild-type and mutant PKCs were immunoprecipitated and remained attached to the Protein A/G agarose beads as before, but the kinase reaction contents could be centrifuged through a streptavidin membrane filter which retained the biotinylated peptide substrate, post-labeling. The ultracentrifugation units could then be washed, and the level of peptide phosphorylation could be measured by adding the units directly to scintillation fluid and reading in a scintillation counter. Not only did this allow for the ability to test any biotinylated target, this also made it possible to quantify the kinase activity and to measure it against "background" from negative controls. The new, speedier protocol also provided the potential for higher throughput, multiple determinations and statistical analysis.

Figure 13 shows the results of one of the first kinase assays performed this way. The substrate used in this case was a "pseudosubstrate" peptide (RFARKGSLRQKNV) designed specifically for the PKC Kinase Assay Kit. It had been reported to be readily phosphorylated *in vitro* by all PKCs [69]. Wild-type PKC-δ and -ε were quite active against pseudosubstrate, while the V5 chimeras were significantly weaker. Importantly, both V5 chimeras were shown to have some kinase activity over the known kinase deficient mutant, PKC-δKD. PKC-δ/εV5 had a mean value of 4511
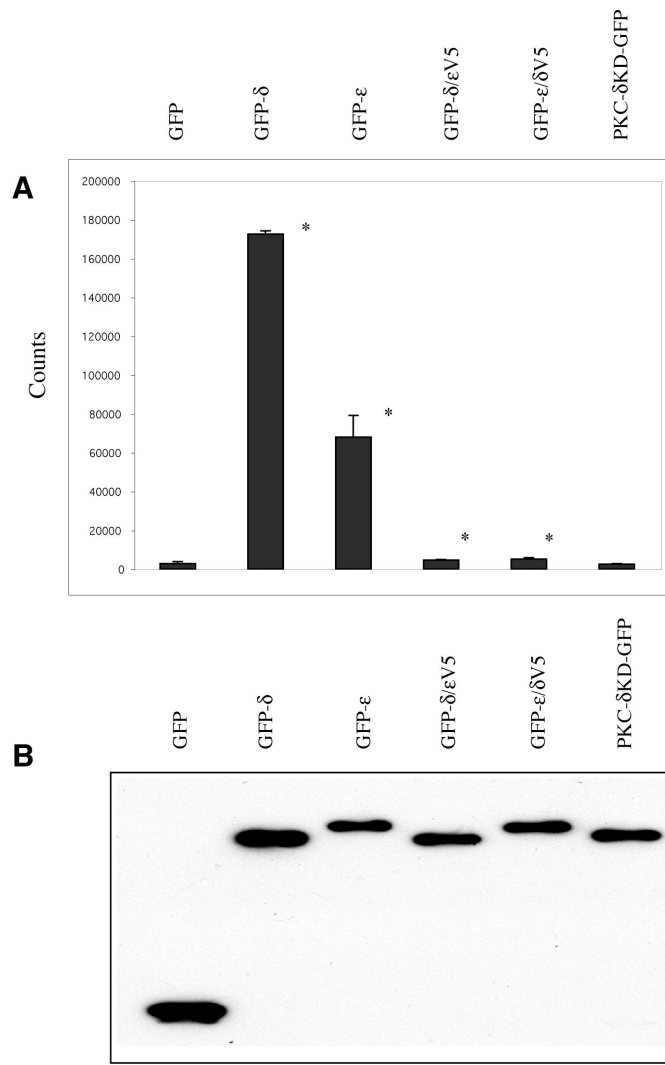
Figure 13. Kinase assay of wild-type and chimeric PKCs against pseudosubstrate peptide. GFP-tagged PKCs were immunoprecipitated from transiently transfected HEK 293 cells with anti-GFP-antibody and Protein A/G agarose beads. The bead-bound enzyme was then allowed to phosphorylate a biotinylated pseudosubstrate peptide (Calbiochem). A. Histogram of mean and standard deviation of scintillation counts from three separate reactions. (* indicates value is significantly different from PKC-δKD-GFP; P≤0.01) B. Western blot of an aliquot of IP product stained with anti-GFP to show relative amount of enzyme used in each reaction.

counts and PKC-ε/δV5 had a mean value of 5108 counts, compared to KD-PKC-δ's mean value of 2559 counts (Figure 13). A t-test, assuming unequal variances, based upon three samples from each grouping proved that the differences between chimeric PKC activity and the kinase activity of the catalytically deficient mutant were indeed significant (P= 0.01 for both chimeras). These results are qualitatively similar to those obtained from assays performed with full-length protein substrate. It therefore seems likely that steric hindrance from full-length protein targets was not a factor in previous assays.

Other peptide substrates were also tested to determine whether exchange of the δ and ε V5 domains had resulted in altered *specificity* as opposed to altered *kinase activity*. Again using the modified Protein Kinase C Assay Kit protocol, V5 chimeric kinase activity was tested against four biotinylated peptide substrates. Based upon their amino acid sequence, three of these peptides, kindly donated by Stephen Shaw, NCI, NIH, were predicted to act either as preferred, neutral or disfavored substrates for PKC-δ. Dr. Shaw has done extensive work on the specificity of PKC isoforms and has established a predictive method for determining whether or not a given peptide is "preferred" or "disfavored" by any given PKC isoform [70]. Figure 14 shows the results of a kinase assay performed with peptides that cover a range of scores in predictive tests for PKC-δ substrate preference: the FLJ20719 peptide (RRGRRSTKKRRR), predicted to be a preferred PKC-δ target, a vimentin peptide (SAVRLRSSVPGV) scoring low in Dr. Shaw's predictive model for PKC-δ specificity, and the BRCA1 peptide (LRKSSTRHIHA) predicted to be intermediate.
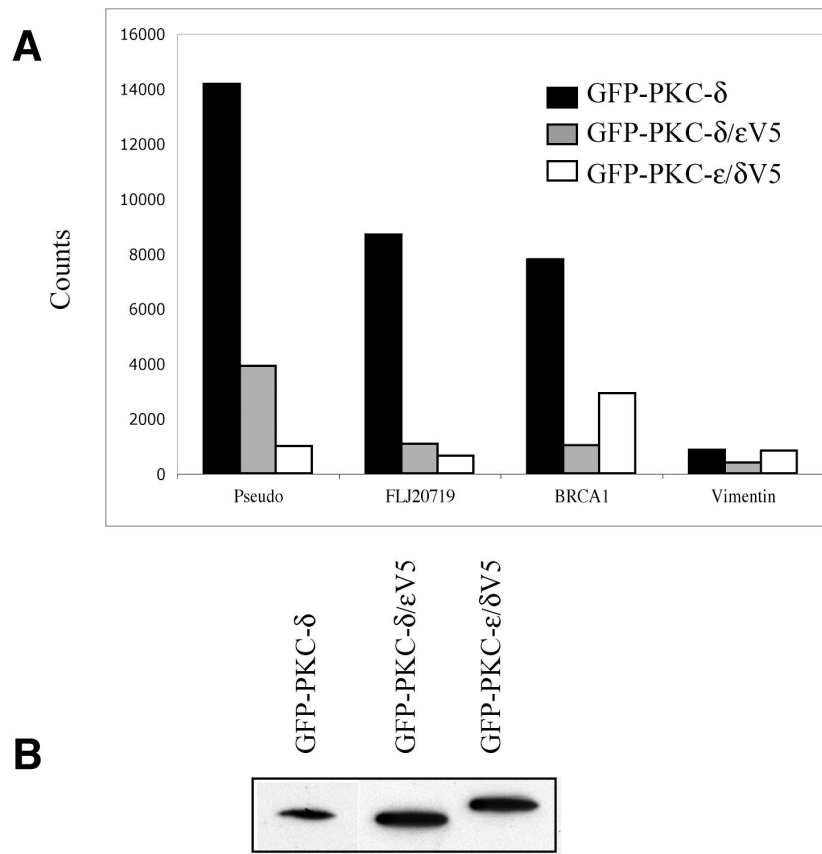
Figure 14. Wild-type and chimeric PKC kinase specificity. GFP-tagged PKCs were immunoprecipitated from transiently transfected HEK 293 cells with anti-GFP and Protein A/G agarose beads. The bead-bound enzyme was then allowed to phosphorylate a biotinylated peptide (Pseudosubstrate, FLJ20719, BRCA1 or Vimentin as indicated below the histogram) in the presence of γ^{32}P-ATP, DAG, Mg^{2+}, PS and cold ATP. A. Histogram of scintillation counts from reactions with different peptide substrates. B. Western blot of an aliquot of IP product stained with anti-GFP to show amount of enzyme used in each reaction.

The pseudosubstrate peptide from the PKC Assay Kit (RFARKGSLRQKNV), already known to be a "preferred" pan-PKC substrate, was also included in this experiment. PKC-δ activity against the pseudosubstrate and FLJ20719 peptides was highest and PKC-δ activity against the vimentin-derived peptide was lowest, in accord with the predicted preference of each substrate. The V5 chimeras showed weak activity against all four peptides. Like PKC-δ, PKC-δ/εV5 showed a slight preference for the pseudosubstrate peptide above the others. PKC-ε/δV5 showed a slight preference for the BRCA1-derived peptide. Because PKC V5 chimera kinase activity seemed to be weak for all peptides as well as for both full length substrates tested and because the pattern of preference for the PKC-δ/εV5 chimera was similar to that PKC-δ, it is unlikely that the weak kinase activity observed with the V5 chimeras in these assays is the result of altered substrate specificity.

PKC Phosphorylation State

If reduced kinase activity in the PKC mutants was not a result of a change in substrate specificity, it might have been the result of a lack of phosphorylation at the phosphorylation priming sites. As mentioned in the Introduction, active PKCs expressed in adherent mammalian cells are generally phosphorylated, to some degree, at all three priming sites. Using antibodies to specific phospho-threonine and phospho-serine residues, the phosphorylation state of the novel PKC mutants was tested. Figure 15 shows that, whereas wild-type PKC-δ is phosphorylated at all three priming sites, PKC-δ/εV5 and -ε/δV5 lack phosphorylation in at least one of the priming sites. PKC
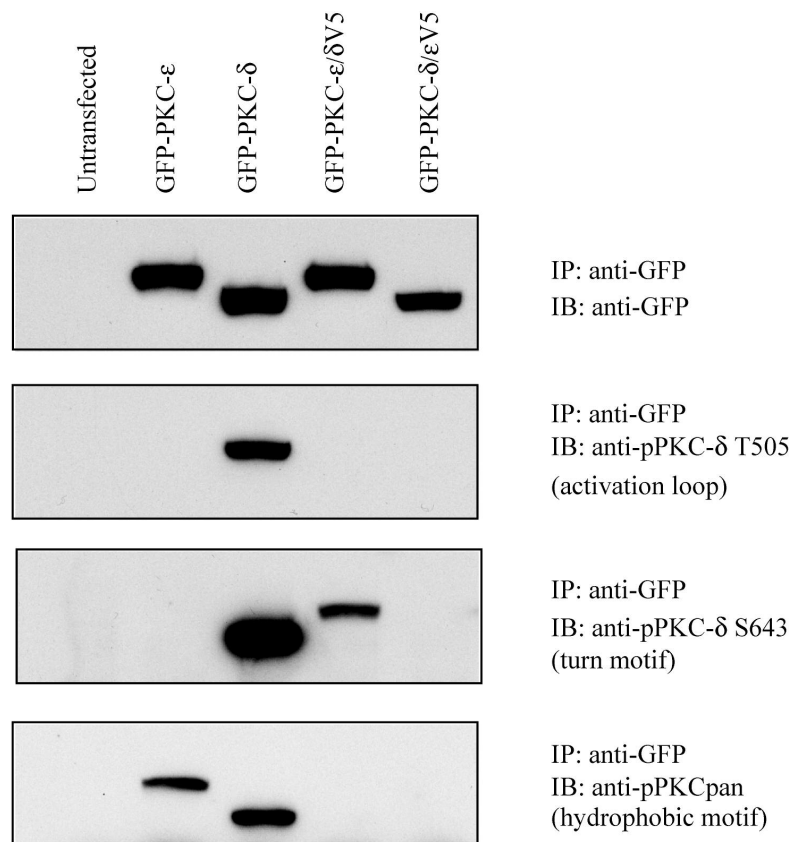
Figure 15. Phosphorylation status of wild-type and chimeric PKC-δ and -ε. GFP-tagged PKCs were immunoprecipitated from transiently-transfected CHO cell lysates using a rabbit anti-GFP antibody and assayed by Western blot. The blot was stained with anti-GFP or phospho-specific antibodies. The anti-pPKCpan antibody recognizes both PKC-δ and PKC-ε phosphorylated at the hydrophobic motif.

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-δ/εV5 is unphosphorylated at the activation loop and the hydrophobic motif; PKC-ε/δV5 is unphosphorylated at the hydrophobic motif site. Although PKC-ε/δV5 is phosphorylated at the turn motif, the level of phosphorylation at this site is reduced. Unfortunately, phospho-specific antibodies for the activation loop and turn motif sites of PKC-ε are not yet available, so the phosphorylation status of those sites within the chimeras remains unknown.

<u>Summary</u>

PKC-δ/εV5 and -ε/δV5 were capable of translocation upon TPA stimulation and both mutants showed significantly reduced activity in *in vitro* kinase assays against multiple peptide substrates, including full length targets such as Histone H1 (Figure 9, Figure 10, and Figure 12) and Myelin Basic Protein (data not shown) as well as peptide substrates derived from PKC pseudosubstrate, BRCA1 and vimentin sequences (Figure 13 and Figure 14). The PKC C-terminal truncation mutants showed little kinase activity against Histone H1 with one exception: PKC-εCD6 (Figure 9, Figure 10 and Figure 12). Results with the PKC-ε truncation mutants suggest that the elimination of even one priming phosphorylation site severely decreased kinase activity, since PKC-εCD6, which retains all three priming sites, consistently showed kinase activity, and the PKC-εCD20 mutant did not (Figure 10). Interestingly, this pattern did not hold for PKC-δ where the PKC-δCD11 mutant, that also retained all three priming sites, displayed little, if any, kinase activity (Figure 9 and Figure 12). It may be that the larger number of amino acids that follow the final priming site in PKC-

δ, compared to PKC-ε (12 vs. 7), adds increased stability to the molecule. Thus, the deletion of 11 PKC-δ V5 amino acids might be more disruptive than the deletion of 6 PKC-ε V5 amino acids, despite the fact that the final priming site is retained in both mutants.

In designing the PKC V5 truncation mutants, we expected to map the truncation length for loss of kinase activity, i.e., we expected at least one of the truncation mutants to show deficient kinase activity. In contrast, the reduced *in vitro* kinase activity of the PKC V5 chimeric mutants was unexpected. The results with the truncation mutants and the prominent role that the phosphorylation priming sites play in V5 domain function by virtue of their clustered location within the V5 domain led us to conclude that the phosphorylation status of these mutants should be addressed. Results with phospho-specific antibodies showed that PKC-δ/εV5 was missing at least two phosphorylations and that PKC-ε/δV5 was unphosphorylated within at least one priming site motif. This lack of phosphorylation provided a means to explain the apparently disparate results witnessed up to this point: the PKC V5 chimeras' ability to translocate and the reduced kinase activity witnessed in the *in vitro* experiments? Further experiments would show whether phosphorylation could be restored to the V5 chimeras, resulting in fully "primed" mutants, and whether kinase activity could be rescued by "priming" PKC mutants either by inducing phoshorylation at unprimed sites or substituting unphoshorylated residues with glutamic acid.

CAN PKC ACTIVITY BE RESCUED IN PKC MUTANTS?

TPA Does Not Induce Phosphorylation at Unprimed Sites in Novel PKC Chimeras

Of the many questions raised by the results of the kinase assays and phosphorylation status experiments, it seemed most logical to start with the following: was it possible to restore mutant PKC phosphorylation status to normal? Parekh *et al.* showed that activation loop and hydrophobic motif phosphorylation of PKC-δ could be induced by the addition of TPA and serum to serum-starved cells and that this phosphorylation was not dependent upon kinase activity of PKC-δ itself [19]. In these experiments serum starvation and placement of HEK293 adherent cells into suspension media was required to induce dephosphorylation of PKC-δ at the activation loop and hydrophobic motif to start.

In our experiments serum starvation was not required to induce dephosphorylation of the V5 chimeras since these sites were already unphosphorylated in the presence of serum. Interestingly, 20 minute TPA stimulation of CHO-K1 cells that overexpressed GFP-PKCs did not induce phosphorylation at the activation loop site of PKC-δ/εV5 or the hydrophobic motif of PKC-ε/δV5 as expected (Figure 16). It did, however, increase the level of phosphorylation at priming sites that were already phosphorylated before addition of TPA. This was true for all *primed* sites in PKC-δ, -ε and -ε/δV5. Thus, it appeared that TPA stimulation was capable of increasing the level of phosphorylation at sites that were already phosphorylated, but phorbol ester was unable to induce *de novo* phosphorylation at unprimed sites.
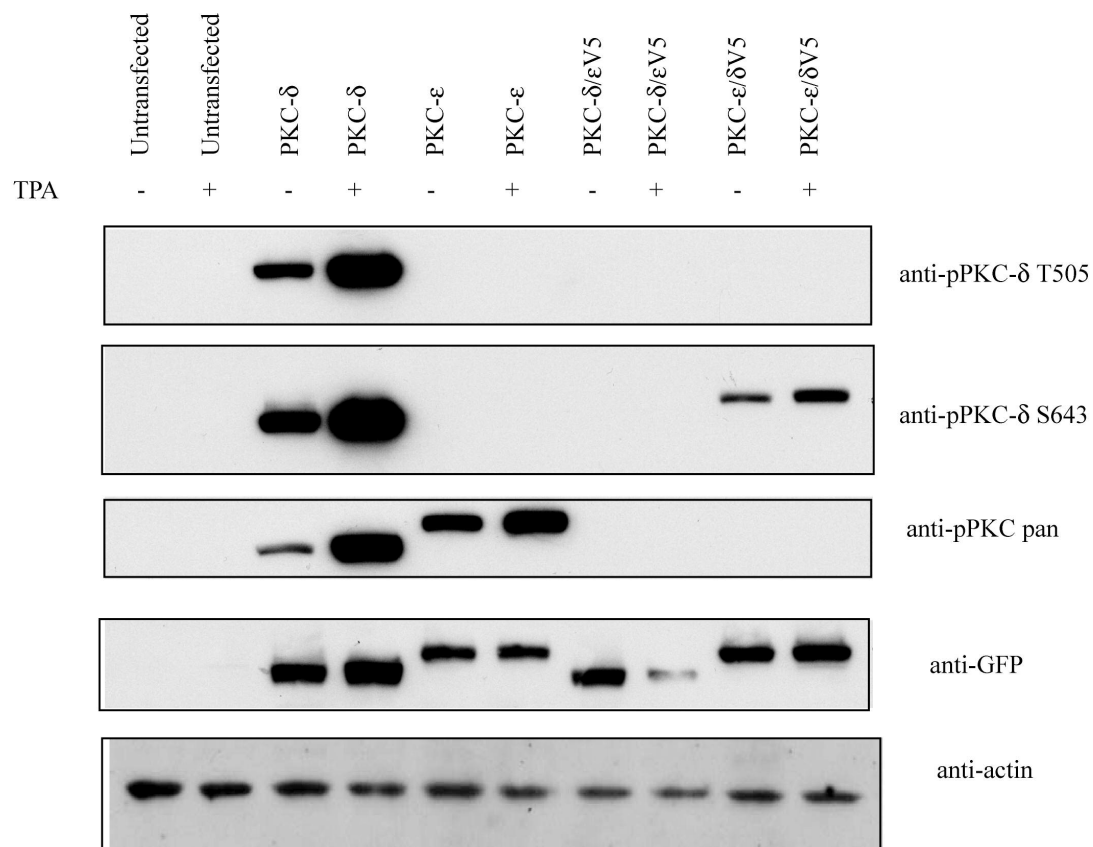
Figure 16. Western blot analysis of TPA-stimulated phosphorylation of wild-type and chimeric novel PKCs. CHO cells were transiently transfected with GFP-tagged PKCs 24 hours before stimulation with 100 nM TPA for 20 min. Cells were lysed and assayed by Western blotting with anti-GFP and phospho-specific antibodies for PKC-δ and -ε priming sites, as indicated. Approximately 15 μg of total cell lysate was loaded in each lane. The anti-actin panel shows that similar amounts of protein were loaded.

<u>Can Glutamic Acid Substitution Rescue PKC Kinase Activity?</u>

Mutation of the activation loop threonine in PKC-βII, PKA or PKB to alanine inhibits the activity of all three kinases, whereas substitution with aspartic acid at this site in PKA and PKB or with glutamic acid in classical isoforms of PKC, to simulate the negative charge resulting from phosphorylation of threonine, resulted in the formation of a catalytically competent kinase [15, 16, 71, 72]. Glutamic acid substitution at the activation loop threonine in PKC-ε also resulted in a kinase-active molecule that was constitutively phosphorylated at the hydrophobic motif priming site [10]. As mentioned in the Introduction, PKC-δ does not require phosphorylation at T505, presumably because a compensatory glutamic acid nearby (E500) compensates for the negative charge requirement [17]. It should be noted, however, that catalytic activity was modest when the activation loop is not phosphorylated [18].

In an attempt to rescue kinase activity of the V5 chimeras and truncation mutants, site-directed mutagenesis was employed to substitute glutamic acid for the unphosphorylated activation loop threonine in those constructs. Additional mutants were produced that also contained a glutamic acid substitution at the unphosphorylated hydrophobic motif priming site within the chimeras, and these constructs were designated DM for "double mutants" (see Table 1). Figure 17 shows the results of kinase assays with a subset of these mutants. Kinase activity was maintained in wild-type PKC-δ with single glutamic acid substitutions either at the activation loop (T505E) or at the hydrophobic motif (S662E) sites, but kinase activity seemed to be even further reduced in the PKC-δ/εV5T505E and DM chimeric mutants compared to

Figure 17. Kinase activity is not rescued by glutamic acid substitution at activation loop/hydrophobic motif priming sites within PKC-δ/εV5. Wild-type PKC-δ and PKC-δ/εV5 were mutated at the activation loop (T505E), at the hydrophobic motif (S662E) or both (DM). These constructs were transiently expressed in HEK293 cells, immunoprecipitated with anti-GFP and Protein A/G agarose beads, and reacted against Histone H1 for 30 min at 30°C as described in Methods and Materials. A. Autoradiograph of radiolabeled Histone H1. B. Western blots of an aliquot of the same immunoprecipitates stained with anti-GFP to show that similar amounts of GFP-PKC constructs had been loaded and phospho-specific antibodies as indicated.

the PKC-δ/εV5 mutant with no S/T -> E substitutions. In addition, the negative charge

at the activation loop of the PKC-δ/εV5 T505E chimera was unable to induce priming

phosphorylation at the hydrophobic motif site. Yet all priming phosphorylations were

maintained in wild-type PKC-δ with single glutamic acid substitutions at the activation

loop and the hydrophobic motif (Figure 17).

Similar results were seen when PKC-ε/δV5 T566E and DM constructs were

tested for activity. Figure 18 shows that PKC-ε T566E retained kinase activity while

the PKC-ε/δV5 T566E lost what activity PKC-ε/δV5 had. The PKC-ε/δV5 DM

construct also has no apparent kinase activity. Interestingly, in both cases where kinase

activity had been lost, the turn motif phosphorylation was also missing. PKC-ε/δV5

T566E was also unphosphorylated at the hydrophobic motif, but the PKC-ε T566E

mutant showed a level of phosphorylation at that site similar to wild-type PKC-ε.

Thus, glutamic acid substitution was not only unable to induce phosphorylation at the

hydrophobic motif of the mutant of the PKC-ε/δV5 T566E mutant, but the turn

phosphorylation originally present in the PKC-ε/δV5 chimera was lost. Thus, the

consequences of glutamic acid substitution at the activation loop of the chimeric

construct are more severe than that for the wild-type PKC. Activation loop glutamic

acid substitution also did not rescue kinase activity of PKC-δCD24 and, instead,

resulted in the loss of phosphorylation at the turn motif site (data not shown).

Since turn motif phosphorylation and kinase activity were both lost in the PKC-

ε/δV5 activation loop and double (activation loop + hydrophobic motif) mutants, it was

possible that this loss of phosphorylation was a major contributor to loss of kinase
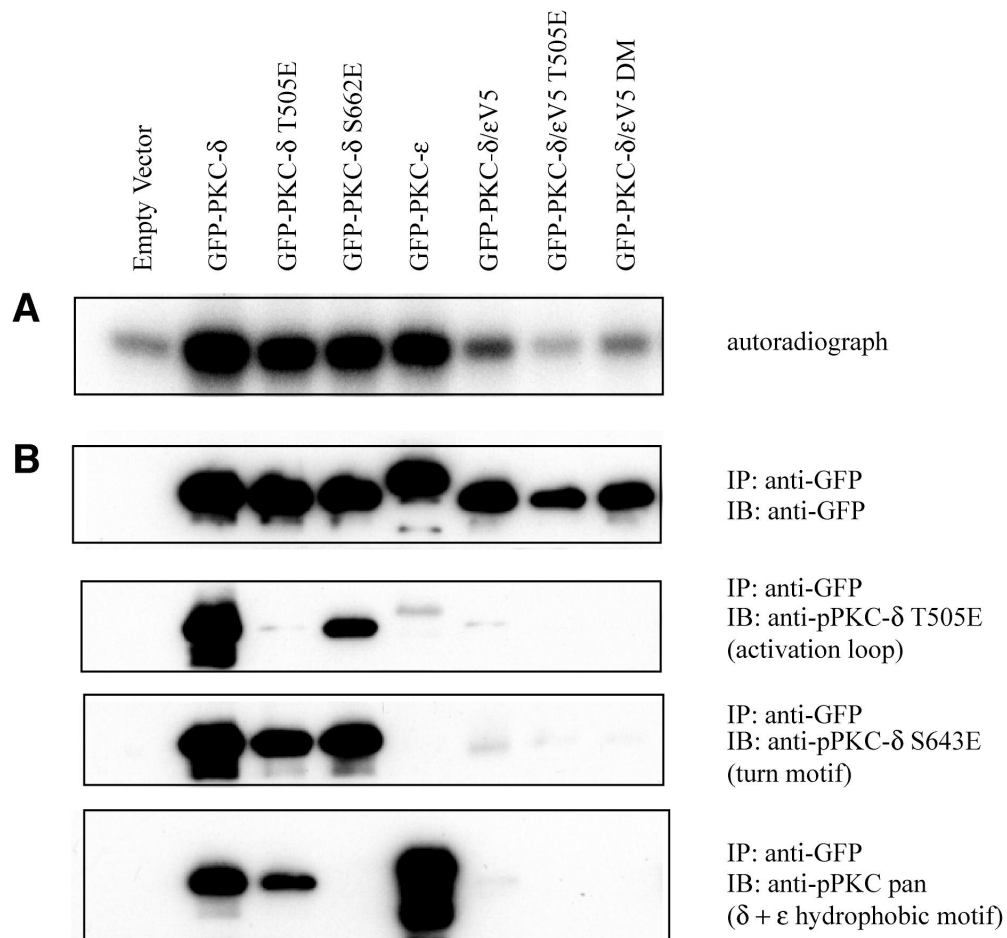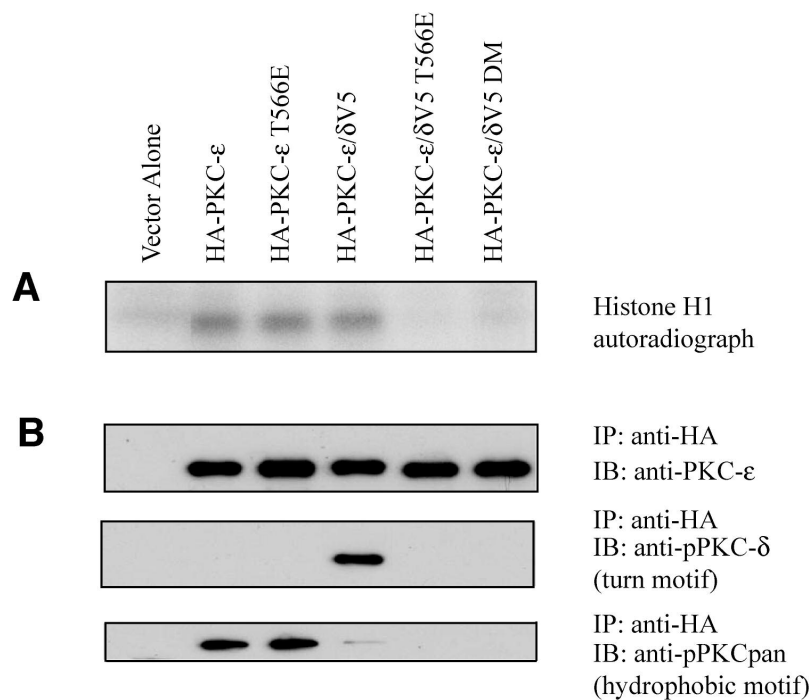
Figure 18. Kinase activity is not rescued by glutamic acid substitution at activation loop/hydrophobic motif priming sites within PKC-ε/δV5. Wild-type PKC-ε was mutated at the activation loop (T566E), and PKC-ε/δV5 was mutated either at the activation loop (T566E) alone or at both T566 and the S662 hydrophobic motif site (DM). These constructs were transiently expressed in HEK293 cells, immunoprecipitated with anti-GFP and reacted against Histone H1 for 30 min at 30°C as described in Methods and Materials. A. Autoradiograph of radiolabeled Histone H1. B. Western blots of an aliquot of the same immunoprecipitates stained with anti-PKC-ε to show that similar amounts of PKC were used in each assay and phospho-specific PKC antibodies as indicated.

activity. PKC-δ and -ε V5 chimeric triple mutants were then utilized to see if glutamic

acid substitution at all three priming sites would be capable of rescuing PKC kinase

activity. Figure 19 shows the results of this work. When performed in triplicate

against pseudosubstrate peptide, PKC-δ and -ε V5 chimeric triple mutants (TM)

showed even less kinase activity than their unsubstituted chimeric constructs. The

mean number of counts for PKC-δ/εV5 and -ε/δV5 were 4206 and 5697, respectively.

In both cases, these values are more than twice the mean of GFP alone (2021) and for

PKC-ε/δV5, this difference in mean is significant (P=0.01). The mean number of

counts for the PKC-δ/εV5TM and -ε/δV5TM were 2038 and 2154, respectively. It was

now abundantly clear that glutamic acid substitution could not rescue kinase activity

within the context of the V5 chimeras.

Lessons from Wild-type PKC-δ Glutamic Acid Mutants

        Glutamic acid substitutions were made within wild-type PKC-δ at the activation

loop (T505E), the turn motif (S643E), and the hydrophobic motif (S662E). In addition,

a double mutant (T505E/S662E) and triple mutant (T505E/S643E/S662E) variant were

also obtained. These constructs were designed as controls for the PKC-δ and -ε V5

chimeric glutamic acid substitution mutants. Yet kinase assays performed with these

constructs alone were instructive. Whereas PKC-δ was able to maintain kinase activity

with a glutamic acid substitution made at any one of the three priming sites,

substitutions made to two or more sites abrogated kinase activity (Figure 20).
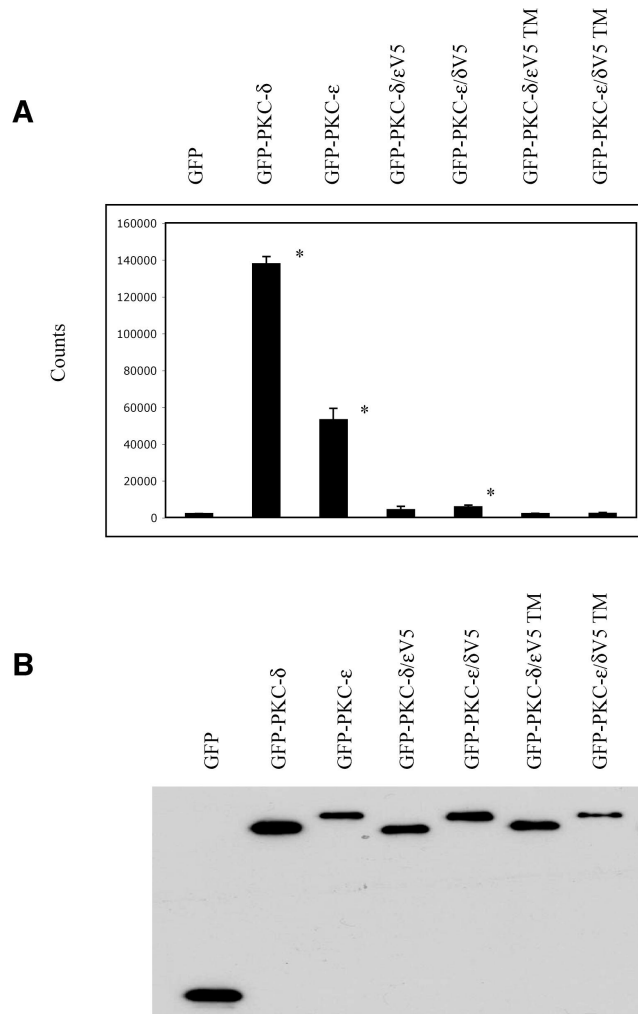
Figure 19. Kinase activity is not rescued by glutamic acid substitution at all three priming sites within PKC-ε/δV5 and -δ/εV5. PKC-ε/δV5 and -δ/εV5 were mutated at the activation loop, the turn motif site and the hydrophobic motif site. These triple mutant (TM) constructs were transiently expressed in HEK293 cells, immunoprecipitated with anti-GFP and reacted against a biotinylated pseudosubstrate peptide for 30 min at 30°C as described in Methods and Materials. A. Means and standard deviations of kinase assay performed in triplicate. (* indicates value is significantly different from cells expressing only GFP; P≤0.01) B. Western blots of an aliquot of the same immunoprecipitates stained with anti-GFP to control for amount of enzyme used in the reaction.
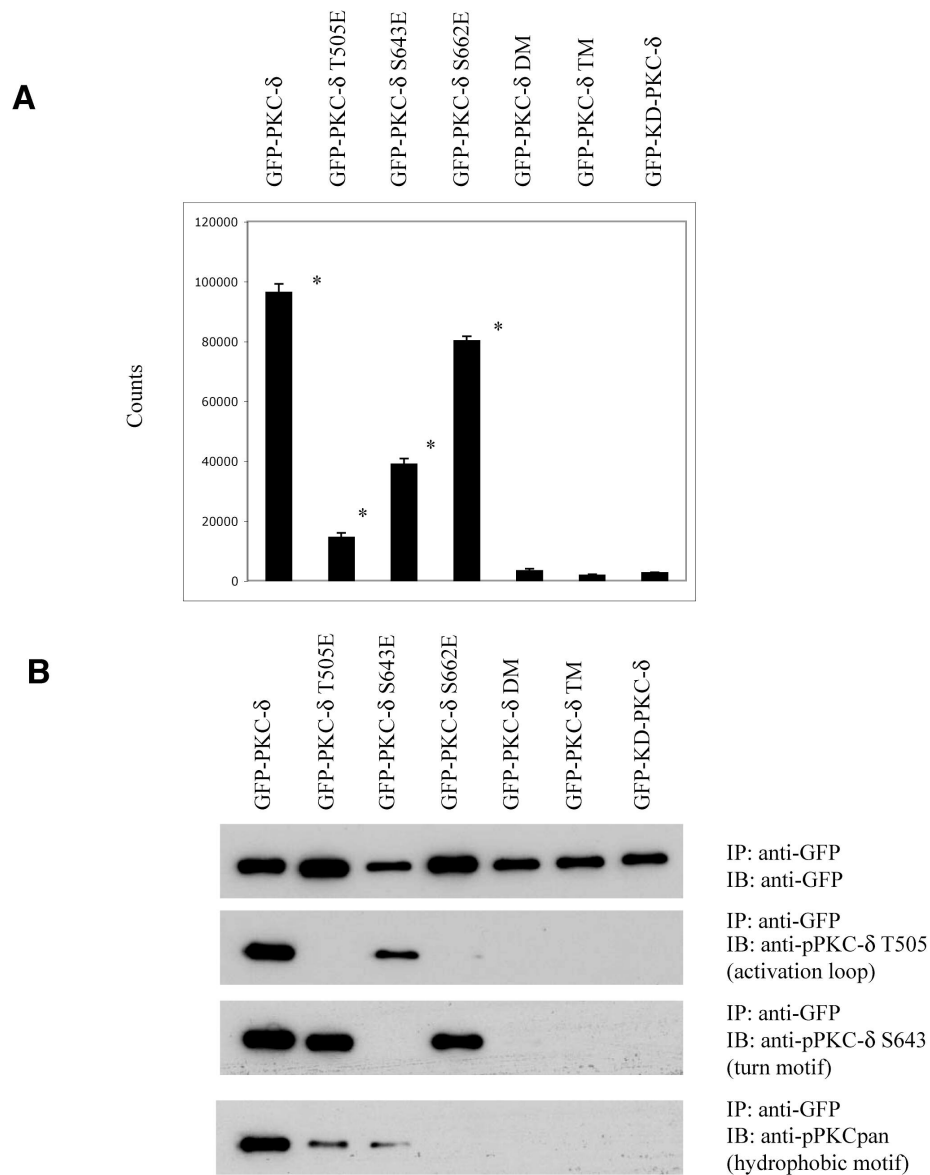
Figure 20. Kinase activity of PKC-δ glutamic acid substitution mutants. PKC-δ was mutated at the activation loop, the turn motif site and the hydrophobic motif site individually, at both the activation loop and hydrophobic motif (DM) and at all three priming sites (TM). PKCs were transiently expressed in HEK293 cells, immunoprecipitated with anti-GFP and reacted against a biotinylated pseudosubstrate peptide for 30 min at 30°C as described in Methods and Materials. (* indicates value is significantly different from control lysates of cells expressing kinase-dead (KD) KD-PKC-δ; P≤0.006) A. Means and standard deviations of kinase assay performed in triplicate. B. Western blots of an aliquot of the same immunoprecipitates stained with anti-GFP (to control for amount of enzyme used in the reaction) and phospho-specific PKC antibodies.

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A correlation of the kinase activity of the substitution mutants with their phosphorylation status proved to be quite intriguing (Figure 20). Single glutamic acid substitutions maintained a fully primed status with the T505E and S643E PKC-δ mutants. However, the PKC-δ S662E mutant lost its priming phosphorylation at the activation loop site with very little consequence in kinase activity. The PKC-δ DM lost its priming phosphorylation at the turn motif as did the PKC-ε/δV5 DM mutant. The results of this experiment showed a clear requirement for negative charge at both V5 priming sites, since without that, kinase activity was lost. The exception is the PKC-δ TM which did not have kinase activity despite the negative charges from glutamic acid residues at the turn and hydrophobic motifs. However, in this case it would appear that the second conclusion from these results supercedes: PKC-δ loses kinase activity when more than one priming phosphorylation site is substituted with a glutamic acid residue. This loss of kinase activity is likely not the result of the missing turn motif phosphorylation, since that is an autophosphorylation site. Rather, the double substitution likely is unable to compensate for the stability provided by the larger phosphate groups, and the loss of phosphorylation at the turn motif is reflective of that fact. It should be noted that a similar experiment performed with PKC-ε T710E (turn motif), PKC-ε DM (T566E/S729E) and PKC-ε TM (T566E/T710E/S729E) also showed that more than one glutamic acid substitution within a wild-type isoform resulted in kinase inhibition (data not shown).

<u>Second Generation PKC-δ and -ε V5 Chimeras</u>

The genomic structure of murine PKC-δ was published in 2003 [73], and although that of PKC-ε has not been published, the predicted exon boundaries for mouse PKC-ε are in perfect alignment with that of PKC-δ. This information revealed that the final exon of each of these isoforms lay slightly 3' of the boundary used as the basis for the original PKC-δ/εV5 and -ε/δV5 constructs. New PKC V5 chimeras were designed and made based upon the available genomic structure of PKC-δ to see if PKC function could be rescued in this manner. These chimeras differ only slightly from those used heretofore, with the C4/V5 boundary newly defined as the final exon for each isoform (Exon 18 for PKC-δ and Exon 23 for PKC-ε). The resulting new chimeras exchange the last 52 and 48 amino acids of PKC-δ and -ε, respectively, and thus represent an overall exchange of less sequence than the original PKC-ε/δV5 and -δ/εV5 chimeras (Figure 21).

When tested for kinase activity, these new chimeras also showed significantly decreased kinase activity compared to wild-type PKC-δ and -ε and levels of activity similar to those of the original PKC-δ and -ε V5 chimeras (Figure 22). The phosphorylation status of these new V5 chimeras was difficult to ascertain; some Western blots revealed low levels of phosphorylation at the activation loop, turn motif and hydrophobic motif but the results were not consistent and could not be correlated to a particular cell type or context (data not shown).

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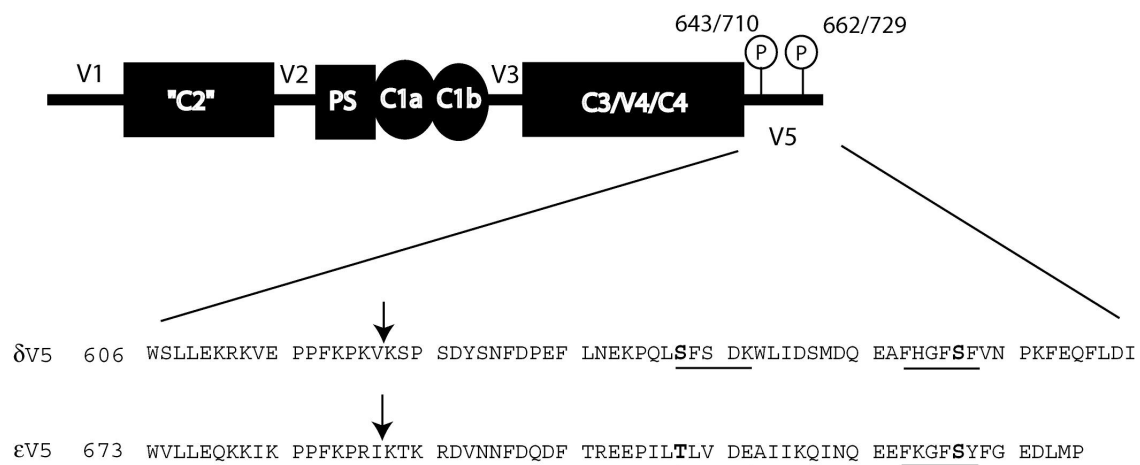
Figure 21. Depiction of the V5 domains of PKC-δ and -ε. Sequences shown are for the V5 domains as defined in the original chimeric constructs, PKC-δ/εV5 and -ε/δV5. Arrows indicate the boundary of the final exon for each isoform. Sequences to the right of the arrows were exchanged in the production of the revised V5 chimeras, PKC-δ/ε23 and -ε/δ18.
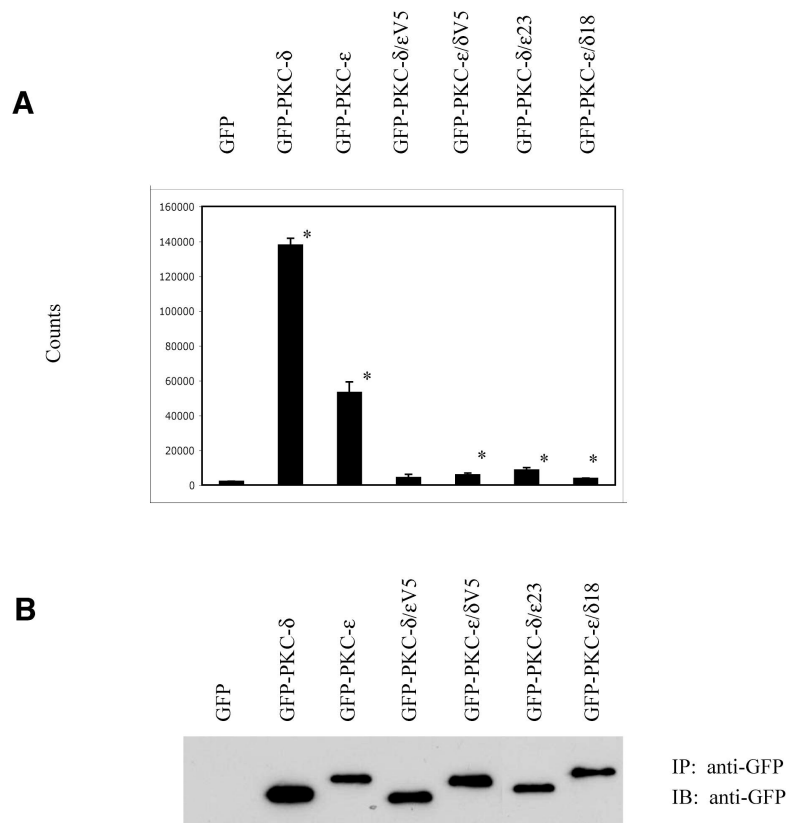
Figure 22. Kinase activity is not rescued by exchange of only final exon of PKC-δ and -ε. New chimeras were constructed in which only the final exon of PKC-δ (Exon 18) and -ε (Exon 23) were exchanged, and these mutants were transiently expressed in HEK293 cells, immunoprecipitated with anti-GFP and protein A/G Sepharose beads and reacted against a biotinylated pseudosubstrate peptide for 30 min at 30°C as described in Methods and Materials A. Means and standard deviations of kinase assay performed in triplicate. (* indicates value is significantly different from results of lysates from cells expressing only GFP; P≤0.03) B. Western blots of an aliquot of the same immunoprecipitates stained with anti-GFP to control for amount of enzyme used in the reaction.

<u>PKC V5 Chimeras Bind to PDK-1</u>

As mentioned in the Introduction, PDK-1 phosphorylates PKCs at the activation loop site. In classical PKCs, this is the first and most important priming phosphorylation event; subsequent priming occurs via autophosphorylation for those isoforms [20, 23]. The role of PDK-1 in priming of novel PKC isoforms is also important, although PKC-δ has been shown to maintain kinase activity even when the activation loop site is not phosphorylated [17]. Activation loop phosphorylation of wild-type PKC-δ increased upon TPA stimulation, but the same did not occur for PKC-δ/εV5 (Figure 16). One possible explanation for the lack of activation loop phosphorylation in the PKC V5 chimera is that PKC-δ/εV5 is unable to bind to PDK-1. This would seem unlikely, since others have shown that PDK-1 could be immunoprecipitated with co-expressed PKC-α, -βI, -δ, -ε, -ι and -ζ in HEK 293 cells [18]. Nevertheless, substrate protein conformation has been reported as a mechanism to regulate PDK-1 phosphorylation [63], and it was possible that PKC-δ and -ε V5 chimeras possessed enough difference in conformation to prevent PDK-1 binding. Figure 23 shows the result of a co-immunoprecipitation experiment performed with the redesigned PKC-δ and -ε V5 chimeras. PKC-δ/ε23 and PKC-ε/δ18, as well as wild-type PKC-δ are able to complex with PDK-1 in CHO-K1 cells upon TPA stimulation (Figure 23), although the V5 of PKC-ε appears to bind PDK-1 less well than that of PKC-δ. Therefore, the lack of activation loop phosphorylation observed in the PKC V5 chimeras was not due to an inability to bind PDK-1.
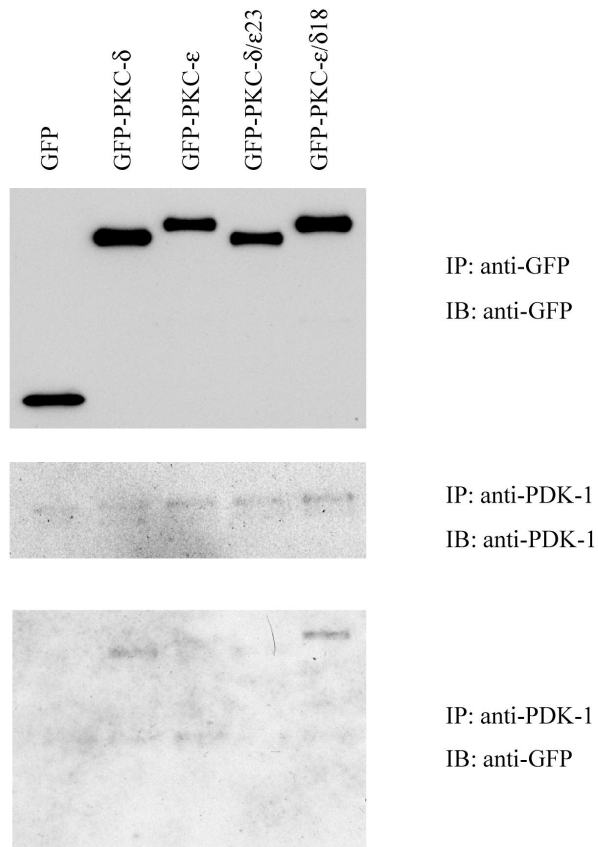
Figure 23. Co-immunoprecipitation of GFP-tagged PKCs with PDK-1. PKCs were transiently expressed in CHO cells that were stimulated with 100 nM TPA for 20 minutes. The cells were lysed and PKCs were immunoprecipitated with anti-PDK-1, anti-GFP and Protein A/G sepharose beads. Western blots show staining with anti-GFP and anti-PDK-1 antibodies.

Summary

Novel PKC V5 chimera kinase activity and phosphorylation status could not be rescued, either by stimulation with TPA (Figure 16), or glutamic acid substitution at unphosphorylated priming sites (Figure 17, Figure 18 and Figure 19). Glutamic acid substitution at priming sites also did not induce phosphorylation at priming sites that originally lacked phosphorylation in the V5 chimeras. Experiments performed with wild-type PKC-δ and -ε glutamic acid mutants revealed that more than one glutamic acid substitution resulted in a kinase-deficient molecule (Figure 20). However, single glutamic acid substitutions at any of the three priming sites within PKC-δ allowed for retention of kinase activity and, usually, retention of a "fully-primed" molecule. Only the PKC-δ S662E mutant demonstrated a lack of phosphorylation at one priming site (the activation loop site), but this did not abrogate kinase activity (Figure 20).

New V5 chimeric constructs, which exchanged Exon 23 of PKC-ε for Exon 18 of PKC-δ, also displayed little difference in kinase activity compared to the original V5 chimeras (Figure 22). In several assays, but not all, the redesigned chimeras showed some phosphorylation at observable priming sites, although their phosphorylation status was not consistent (data not shown). Nevertheless, PKC-δ/ε23 and -ε/δ18 were shown to complex with PDK-1 in a co-immunoprecipitation experiment (Figure 23). This indicates that the PKC V5 chimeras maintain proper conformation for PDK-1 binding and suggests that the lack of phosphorylation--at least at the activation loop priming site--within these mutants is a consequence of increased phosphatase activity.

Table 3 summarizes the results of all the kinase assays and the phosphorylation status of those mutants tested in this project.

Table 3. A summary of the phosphoryaltion status and *in vitro* kinase activity of the wild-type and mutant PKCs used in this study. ND, not determined; E, glutamic acid substituted; NA, phospho-specific antibodies not available for that site.

PKC Construct	Activation Loop	Turn Motif	Hydrophobic Motif	Kinase Activity
PKC-δ	+++	+++	+++	+++
PKC-ε	NA	NA	++	++
PKC-δ/εV5	-	NA	-	Weak
PKC-ε/δV5	NA	++	-	Weak
PKC-δ CD11	-	+	ND	-
PKC-δ CD24	-	+	Deleted	-
PKC-δ CD54	-	Deleted	Deleted	-
PKC-ε CD6	NA	NA	ND	+
PKC-ε CD20	NA	NA	Deleted	-
PKC-ε CD50	NA	Deleted	Deleted	ND
PKC-δ/ε23	Varies	NA	Varies	Weak
PKC-ε/δ18	NA	++	Varies	Weak
PKC-δ T505E	E	++	++	+
PKC-ε T566E	E	NA	+	Weak
PKC-δ/εV5 T505E	E	-	-	-
PKC-ε/δV5 T566E	E	-	-	-
PKC-δ S643E	+	E	+	+
PKC-ε T710E	NA	E	ND	+
PKC-δ S662E	-	++	E	+
PKC-ε S729E	NA	NA	E	ND
PKC-δ DM	E	-	E	-
PKC-ε DM	E	NA	E	-
PKC-δ/εV5 DM	E	NA	E	-
PKC-ε/δV5 DM	E	-	E	-
PKC-δ TM	E	E	E	-
PKC-ε TM	E	E	E	-
PKC-δ/εV5 TM	E	E	E	-
PKC-ε/δV5 TM	E	E	E	-

DISCUSSION

<u>The Critical Role of the V5 Domain in Isoform-Specific Function</u>

The V5 domain was shown to be essential for PKC function: the exchange of the V5 region abrogated PKC-δ's ability to mediate macrophage differentiation. However, PKC-δ and -ε V5 chimeras were unable to demonstrate the conferral of isoform-specific function in the 32D differentiation assay. Nevertheless, this establishes the V5 domain as essential in this PKC isoform-specific function. Subsequent experiments sought to elucidate the structures and and mechanisms that led to these results. Translocation studies performed with NIH 3T3 cells showed that the V5 domain is important for PKC subcellular targeting. In this case, both PKC-δ/εV5 and -ε/δV5 mimicked PKC-δ's pattern of translocation to both the plasma and nuclear membranes. Although PKC translocation is stimulus and isoform-specific, it seem reasonable to assume that the translocation pattern for wild-type PKC-δ is similar in NIH 3T3 and 32D cells for two reasons. First, the lipophilicity of TPA drives PKC to membrane organelles. Second, TPA stimulation of PKC-δ has shown consistent translocation to both the plasma and nuclear membranes in other cell types, including A7r5 smooth muscle cells [74] and CHO-K1 cells [75]. Therefore, if translocation were *the* determinant of PKC biological function in this assay, one would predict that both V5 chimeras would be capable of inducing macrophage differentiation in 32D cells. Kinase assays revealed that the V5 domain was also critical for catalytic function. This suggests that differences in PKC-δ and -ε translocation patterns are not

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involved in their isoform specific function in this case and that translocation does not account for the lack of differentiation observed in 32D cells overexpressing PKC-δ and -ε V5 chimeras. It also makes the point that translocation can occur in kinase-deficient PKCs [74], and it underscores the error in the earlier assumption that translocation of a particular PKC construct is a reliable indication of kinase activity.

Differences in expression levels between overexpressed wild-type and chimeric PKCs also do not account for the lack of 32D cell differentiation observed. Two pieces of evidence substantiate this claim. First, PKC-δ/εV5-overexpressing clones were produced that did, in fact, have expression levels equal to that of wild-type PKC-δ (Figure 4), and these clones were unable to induce 32D cell differentiation upon TPA stimulation (Figure 6). Second, it was possible to observe TPA stimulated macrophage differentiation when varying levels of wild-type PKC-δ were overexpressed in the 32D cells. Thus, although overexpression of PKC-δ is required for this assay, the level of PKC-δ overexpression seems not to be critical.

We showed earlier that apoptosis could be mediated by PKC-δ in A7r5 cells, even if a kinase-deficient PKC-δ mutant was utilized, as long as translocation could be achieved [74]. Monocyte differentiation, however, does not work the same way. Li *et al.* showed that an ATP-binding-deficient PKC-δ construct (K376R) was not capable of inducing 32D cell differentiation [68]. It therefore seems most likely that the reduced kinase activity of the V5 chimeras (Figure 9, Figure 10 and Figure 13) resulted in the inability of one or both of the V5 chimeras to induce TPA-stimulated 32D cell differentiation.

These results contrast with those reported for a PKC-α/βIIV5 chimeric mutant that demonstrated the PKC-βII-specific ability to translocate to the nucleus and phosphorylate lamin B [54]. In addition, numerous experiments have been conducted on PKC-βI and -βII, two splice variants that differ only in their V5 domain. These two isoforms are known to translocate to distinct subcellular locations when activated in the same cell type [55, 56] and are associated with different physiological functions [57]. In all of these cases, PKC isoform-specific function was conferred by the V5 domain. It is important to note that, in these biological assays, PKC catalytic activity directly was not measured directly, and it is not certain that the biological functions require active kinases.

Although the reduced kinase activity of the PKC-δ and -ε V5 chimeras is likely to be involved in the lack of isoform-specific function conferral observed in the 32D cell differentiation assay, it is not *necessarily* responsible for this phenomenon. The kinase assays presented here were performed with immunoprecipitated PKCs still tethered to the Protein A/G agarose-antibody complex. This bead-bound form of the enzyme was much less active than recombinant PKC-δ and -ε used as positive controls. Thus, the results of the kinase assays presented here give an indication of the activity of these enzymes but do not reflect their *in vivo* catalytic competence. Even so, both the original set of PKC V5 chimeras and the redesigned, second generation versions were proven to be significantly more active than a kinase-deficient PKC-δ mutant or the GFP-only control (Figure 13 and Figure 22). Therefore, it is possible that both chimeras were active enough *in vivo* to induce differentiation.

The incomplete conferral of isoform-specific function could instead have been due to altered binding specificity of the V5 chimeras or a combination of altered binding specificity with reduced kinase activity. Although the kinase experiment results showed no change in substrate specificity, only five different substrates were tested. Moreover, PKCs can form complexes with many binding partners in addition to their substrate targets, including RACKs and other adapter proteins important for their function. Changes in binding specificity or affinity with these other proteins could explain how PKC-ε/δV5 translocation could mimic that of wild-type PKC-δ, but macrophage differentiation was still not induced. It is also possible that overexpression of PKC-δ in a given cell context leads to a concomitant change in the level of expression and/or activity of other endogenous PKC isoforms and that this change is not induced by PKC-ε/δV5 or by PKC-δ/εV5. Our limited knowledge of PKC binding partners in assays employed here and the critical role of protein-protein interactions in many signaling pathways make dissection of each of these possibilities a daunting— but very important—task for the future.

Lessons from PKC-δ and -ε Truncation Mutants

Experiments performed with PKC truncation mutants showed that an intact and functional V5 domain is essential to PKC-δ and -ε activity: loss of as few as 11 carboxyl terminal amino acids led to abrogated kinase activity in PKC-δ and loss of six carboxyl terminal amino acids in PKC-ε led to reduced *in vitro* kinase activity. These results are in agreement with a study done by Su, *et al.* in which a series of PKC-α C-

terminal deletion mutants revealed that deletion of 15 or more amino acids resulted in partial or total loss of biological activity, whereas deletion of the most carboxyl 11 amino acids resulted in no loss of biological activity [52]. In all cases, deletion of even a small portion of the hydrophobic motif resulted in partial or total loss of activity. The study revealed, however, that even shorter deletions that leave the hydrophobic motif intact can have an adverse effect on PKC function. Both PKC-εCD6 and PKC-δCD11 showed reduced *in vitro* kinase activity and decreased levels of overexpression compared to their wild-type equivalents, despite the retention of the complete hydrophobic motif.

Deletion of even a small portion of the carboxyl terminus of these enzymes likely resulted in substantially disrupting--if not abrogating--phosphorylation at the hydrophobic motif priming site. Because the epitope for the phospho-specific PKC "pan" antibody used to detect phosphorylation at this site is not exactly known, it is impossible to say what the phosphorylation status at that site is in the PKC-δCD11 and -εCD6 mutants. At the time when the C-terminal truncation mutants were designed for the present study, inclusion of the serine target in the hydrophobic phosphorylation site was the main consideration. This study shows for the first time that the amino acids C-terminal to the hydrophobic motif are critical to PKC-δ and -ε function.

The importance of priming phosphorylations in PKC function, including kinase activity and downregulation, is well established [15, 22, 76, 77]. Loss of phosphorylation at even one of the three phosphorylation priming sites could be responsible for the loss of kinase activity and the difficulty in achieving equal levels of expression between wild-type and truncated PKC mutants.

The Critical Role of Priming Phosphorylation in PKC-δ and -ε Function

The role of priming phosphorylations in novel PKC maturation and function remains complex and a matter of controversy. This work confirmed the importance of each of the V5 priming sites in novel PKC-δ and ε function, for without a negative charge at each of the V5 priming sites, kinase activity was significantly inhibited. Yet the requirement for kinase activity seems to be more than just a negative charge at PKC phosphorylation priming sites. Evidence for this includes the following: 1) PKC-δ and -ε TM constructs were both kinase inactive despite the fact that wild-type PKC-δ retained kinase activity with a single glutamic acid substitution at any one the priming sites, 2) Glutamic acid substitutions within the context of the PKC-δ and -ε V5 chimeras were unable to rescue reduced kinase activity. Of course, based upon the abrogated kinase activity of wild-type PKC-δ and -ε that contained glutamic acid substitutions at more than one priming site, one can not expect that multiple glutamic acid substitutions would be able to rescue the kinase activity of the novel PKC V5 chimeras.

Rescue of *in vitro* kinase activity by glutamic acid substitution at unphosphorylated priming sites would have left little doubt as to the cause of the observed reduction in activity of the V5 chimeras and truncation mutants. Unfortunately we now know from our experiments with multiple S/T->E substitutions within wild-type PKC isoforms that we could not have expected more than one glutamic acid substitution in PKC-δ and -ε V5 chimeras to rescue *in vitro* kinase activity. It remains unknown whether these constructs are able to retain/regain some *in*

vivo function despite their abrogated kinase activity. Without the certainty that a positive rescue result would have provided, we are left to speculate about the role of the phosphorylation priming sites in these constructs. The question remains as to whether or not the lack of phosphorylation at these sites is symptomatic of their reduced *in vitro* kinase activity or a cause of it.

More intriguing is the question of why PKC V5 chimeras lack priming phosphorylations at all. If PKC priming begins with binding of PDK-1 to the unphosphorylated hydrophobic motif, and if PDK-1 is indeed the upstream kinase for both PKC-δ and -ε, as has been deduced from PKC/PDK-1 binding experiments and kinase assays, there seems to be no reason for the chimeras to lack phosphorylation at the activation loop site. This study showed that PDK-1 does indeed bind to at least one of the V5 chimeras (Figure 23). Co-immunoprecipitation of GFP-tagged PKCs with an anti-PDK-1 antibody showed that PDK-1 bound to wild-type PKC-δ and -ε as well PKC-ε/δ18 and -δ/ε23. These results suggest that lack of phosphorylation at the activation loop site is not a result of the inability of PKC V5 chimeras to bind to PDK-1. Instead, future investigations should focus on potential changes in phosphatase activity and the access of phosphatases to the activation loop site in these mutants.

Summary

      It is abundantly clear that the V5 domain is essential to nPKC-δ and -ε function. Even very short truncations to the carboxyl terminus of PKC-δ and -ε result in the loss of stable expression and a significant reduction in kinase activity *in vitro*. Yet while this loss of function is easy to observe, rescue of function by addition of the V5 domain from the reciprocal isoform is not easily achieved: PKC-δ and -ε V5 chimeras are also more difficult to express stably and have significantly reduced kinase activity *in vitro*. Nevertheless, PKC-ε/δV5 and -δ/εV5 remained capable of translocating upon TPA retain some statistically significant kinase activity. PKC-δ and -ε V5 chimeras are valuable models for the study of differences in PKC maturation and activation via phosphorylation and protein-protein interactions.

<u>Conclusions</u>

1. The V5 domain is essential to PKC function.

2. Exchange of PKC-δ and -ε V5 domains is enough to abrogate isoform-specific function, but alone it is not sufficient to confer complete isoform-specific function.

3. The V5 domain is clearly involved in modulation of PKC translocation and kinase activity.

4. PKC V5 chimeras lack phosphorylation at critical priming sites, including the activation loop site.

5. This lack of phosphorylation is likely not due to an inability to bind PDK-1 as PKC-δ and -ε V5 chimeras were able to co-immunoprecipitate with PDK-1 after TPA stimulation.

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