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A novel phosphorylation site at Ser¹³⁰ adjacent to the pseudosubstrate domain contributes to the activation of protein kinase C- δ

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Abstract

Protein kinase C- δ (PKC δ) is a signalling kinase that regulates many cellular responses. Although most studies focus on allosteric mechanisms that activate PKC δ at membranes, PKC δ also is controlled via multi-site phosphorylation [Gong et al. (2015) Mol. Cell. Biol. **35**, 1727–1740]. The present study uses MS-based methods to identify PKC δ phosphorylation at Thr⁵⁰ and Ser⁶⁴⁵ (in resting and PMA-treated cardiomyocytes) as well as Thr³⁷, Thr³⁸, Ser¹³⁰, Thr¹⁶⁴, Thr²¹¹, Thr²¹⁵, Ser²¹⁸, Thr²⁹⁵, Ser²⁹⁹ and Thr⁶⁵⁶ (as sites that increase with PMA). We focused on the consequences of phosphorylation at Ser¹³⁰ and Thr¹⁴¹ (sites just N-terminal to the pseudosubstrate domain). We show that S130D and T141E substitutions co-operate to increase PKC δ 's basal lipid-independent activity and that Ser¹³⁰/Thr¹⁴¹ di-phosphorylation influences PKC δ 's substrate specificity. We recently reported that PKC δ preferentially phosphorylates substrates with a phosphoacceptor serine residue and that this is due to constitutive phosphorylation at Ser³⁵⁷, an ATP-positioning G-loop site that limits PKC δ 's threonine kinase activity [Gong et al. (2015) Mol. Cell. Biol. **35**, 1727–1740]. The present study shows that S130D and T141E substitutions increase PKC δ 's threonine kinase activity indirectly by decreasing G loop phosphorylation at Ser³⁵⁷. A S130F substitution [that mimics a S130F single-nt polymorphism (SNP) identified in some human populations] also increases PKC δ 's maximal lipid-dependent catalytic activity and confers threonine kinase activity. Finally, we show that Ser¹³⁰/Thr¹⁴¹ phosphorylations relieve auto-inhibitory constraints that limit PKC δ 's activity and substrate specificity in a cell-based context. Since phosphorylation sites map to similar positions relative to the pseudosubstrate domains of other PKCs, our results suggest that phosphorylation in this region of the enzyme may constitute a general mechanism to control PKC isoform activity.

Keywords

phosphorylation; protein kinase C-delta; pseudosubstrate domain; single nucleotide polymorphism

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AUTHOR CONTRIBUTION

Susan Steinberg conceived the study. Ronald Holewinski performed MS studies. Jianli Gong performed mutagenesis, protein expression, IVKAs and cell-based studies. All authors reviewed/interpreted data and contributed to the final manuscript.

INTRODUCTION

Protein kinase C δ (PKC δ) is a novel PKC (nPKC) family member that plays a key role in signal transduction pathways that regulate a wide range of cellular responses [1,2]. PKC δ is structurally characterized by a C-terminal serine/threonine kinase domain and an N-terminal regulatory region consisting of tandem C1A/C1B domains that bind lipid cofactors and a C2 domain that functions as a protein–protein interaction module. PKC δ also contains an auto-inhibitory pseudosubstrate motif just N-terminal to its C1A domain. This sequence, which resembles a PKC substrate with an alanine substitution at the phosphoacceptor site (P-site), maintains the enzyme in a closed/inactive conformation.

PKC δ is recruited to membranes by growth factor receptors that activate phospholipase C and promote the accumulation of diacylglycerol, a lipid cofactor that interacts with the C1 domain and stabilizes the enzyme in an active conformation at membranes. PKC δ also is directly activated by C1 domain-interacting tumour-promoting phorbol esters such as phorbol PMA. However, mechanisms that allosterically activate PKC δ at membranes do not adequately explain the full repertoire of PKC δ 's cellular activities. Rather, recent studies indicate that phosphorylations at various amino acid residues strategically located throughout the enzyme play specific roles to regulate enzyme stability and/or catalytic activity. For example, PKC δ contains 'priming' phosphorylation sites in the kinase domain activation loop (Thr⁵⁰⁵) and C-tail (Ser⁶⁴³/Ser⁶⁶²; numbering throughout the manuscript is based upon the rodent sequence). These phosphorylation sites are flanked by motifs that are highly conserved across PKC family members and other AGC family kinases. Priming phosphorylations typically are stable (phosphatase-resistant) modifications that convert nascent PKCs into mature catalytically-competent enzymes that reside in the cytosol and are poised to be allosterically activated by lipid cofactors [3]. PKC δ undergoes priming phosphorylations like other PKC family enzymes, but it is unique in that it is catalytically active even without activation loop (Thr⁵⁰⁵) phosphorylation. Rather, PKC δ -Thr⁵⁰⁵ phosphorylation is a dynamically regulated autocatalytic reaction that 'fine-tunes' PKC δ 's catalytic function toward selected protein substrates [4].

Recent studies implicate another phosphorylation site at Ser³⁵⁷ at the tip of PKC δ 's glycine-rich ATP-positioning loop (G-loop, also known as the phosphate-binding P-loop) as another modification that dynamically regulates catalytic activity [5]. Ser³⁵⁷ sits in a highly conserved phosphorylation motif at the entrance to the ATP-binding cleft in close proximity to the P-site on substrates. Our recent studies indicate that PKC δ is recovered from unstimulated cardiomyocytes as a Ser³⁵⁷-phosphorylated enzyme and that Ser³⁵⁷ phosphorylation limits PKC δ 's threonine kinase activity (i.e. the Ser³⁵⁷-phosphorylated enzyme shows a strong preference for substrates with a serine residue at the P-site [5]). Oxidative stress triggers a tyrosine phosphorylation-dependent (C2 domain-driven) mechanism that results in Ser³⁵⁷ dephosphorylation. Ser³⁵⁷ dephosphorylation leads to two changes in PKC δ activity. Ser³⁵⁷ dephosphorylation enhances PKC δ 's lipid-independent activity, generating an enzyme that can phosphorylate substrates throughout the cell, not just on lipid membranes. Ser³⁵⁷ dephosphorylation also confers a high level of threonine kinase activity, enabling PKC δ to phosphorylate a broader range of substrates with either serine or threonine residues at the P-site.

PKC δ contains yet additional phosphorylation sites at more variable regions of the enzyme that are not conserved across other PKC family members. Phosphorylation at these other sites has been viewed as a marker and/or regulator of enzyme activity. For example, we and others identified a cluster of autophosphorylation sites in the variable V3 hinge region of human PKC δ at Thr²⁹⁵, Ser²⁹⁹, Ser³⁰² and Ser³⁰⁴ (Ser³⁰² and Ser³⁰⁴ are not conserved in rodent PKC δ [6,7]). Autophosphorylations at Thr²⁹⁵ or Ser²⁹⁹ have been used as convenient ‘read-outs’ of PKC δ activation [6,8]; mutagenesis studies also indicate that Thr²⁹⁵ phosphorylation is required for maximal PKC δ activity [6]. PKC δ contains another phosphorylation site in its C1A–C1B interdomain region at Thr²¹⁸ [6]. The C1A–C1B interdomain appears to be a phosphorylation ‘hot spot’ since this specific phosphorylation motif is conserved in the C1A–C1B interdomain of PKC θ (where it is required for proper PKC θ membrane recruitment and T-cell antigen receptor signalling responses [9]) and phosphorylation sites in different motifs are found in the C1A–C1B interdomains of PKC ϵ and other PMA–DAG-sensitive kinases such as PKD1 and PKD3 [10–14]. Finally, PKC δ contains a phosphorylation site at Thr¹⁴¹, a site strategically positioned just N-terminal to the pseudosubstrate domain [6]. We previously reported that PKC δ is phosphorylated at Thr¹⁴¹ *in vivo* in cardiomyocytes and that a T141A substitution slows and a T141D substitution accelerates, the tempo of PMA-dependent PKC δ down-regulation [6]. These results indicate that a negative charge due to phosphorylation at Thr¹⁴¹ disrupts an intramolecular interaction between the pseudosubstrate motif and the catalytic pocket and favours PKC δ activation. The present study identifies Ser¹³⁰ as another regulatory phosphorylation site adjacent to the pseudosubstrate domain that cooperates with Thr¹⁴¹ to control the activation of PKC δ .

MATERIALS AND METHODS

Adenoviral infection of cardiomyocyte cultures

Cardiomyocytes were isolated from the hearts of 2-day-old Wistar rats by a trypsin dispersion procedure followed by differential attachment procedures and irradiation to enrich for cardiomyocytes as described previously [15]. Cardiomyocytes grown for 5 days in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum were infected with an adenoviral vector that drives expression of wild-type (WT) PKC δ according to protocols published previously [15]. Cell lysates were prepared 24 h later, following a 30 min treatment with vehicle or 200 nM PMA.

Trypsin digestion and titanium dioxide enrichment

Lysates from resting and PMA-treated cardiomyocytes were prepared in 200 μ l of 8 M urea, 1% SDS with brief sonication and protein concentration was determined by the BCA method. Each sample was treated as follows: 900 μ g of total protein in a solution containing 50 mM NH₄HCO₃, pH 8, 1.6 M urea, 0.2% SDS, 11 mM DTT was incubated at room temperature for 1 h after which samples were treated with iodoacetamide to a final concentration of 30 mM and incubated at room temperature for 1 h in the dark. Samples were treated with 20 μ g of trypsin (Promega) and incubated for 2 h with shaking at 37°C after which point an additional 20 μ g of trypsin was added and the reaction was incubated for an additional 16 h at 37°C with shaking. After 16 h the reaction was stopped by the

addition of 100 μ l of 10% trifluoroacetic acid (TFA) to drop the pH below 2. Samples were desalted on 30 mg Oasis HLB (hydrophilic lipophilic balanced) cartridges (Waters) and eluted in 1 ml of 70% acetonitrile (ACN), 7 mM KH_2HPO_4 , pH 2.65, followed by strong cation exchange (SCX)-fractionation with titanium dioxide (TiO_2) enrichment (87.5%) or TiO_2 (12.5%) enrichment alone.

SCX fractionation

Peptide samples were fractionated by SCX using a variation of the method by Dephoure and Gygi [16]. Briefly, SCX cartridges were prepared by adding 100 mg of SCX bulk medium (polysulfoethyl aspartamide, Nest Group) suspended in 1 ml of 30% ACN to an empty 1-ml cartridge (Applied Separations), allowing the slurry to settle and sealing the column bed with an additional frit. The column was wet with 3 ml of 80% ACN, followed by a 3-ml H_2O wash and then equilibrated with 6 ml of SCX buffer A (of 30% ACN, 7 mM KH_2HPO_4 , pH 2.65). Samples in 70% ACN, 7 mM KH_2HPO_4 , pH 2.65, were loaded on the columns and the flow through was collected. Columns were washed two times with SCX buffer A and flow through was combined with the sample flow through (fraction 1). Samples were eluted with 1 ml of SCX buffer A containing two different salt concentrations (40 mM KCl fraction 2; 150 mM KCl fraction 3). All three fractions were concentrated to approximately 100 μ l and treated with 1 ml of 0.1% FA (formic acid). Samples were desalted using 10 mg of HLB cartridge and peptides were eluted in 300 μ l of 80% ACN, 5% TFA, 1 M glycolic acid buffer and enriched by TiO_2 as described below.

TiO_2 enrichment

The 12.5% of sample not fractionated by SCX was treated with 175 μ l of 80% ACN, 5% TFA, 1 M glycolic acid. All samples (unfractionated and SCX fractions) were treated with 50 μ l of TiO_2 slurry (30 mg/ml in 80% ACN, 5% TFA, 1 M glycolic acid) and incubated overnight with shaking at room temperature. Samples were washed three times with 400 μ l of 80% ACN, 5% TFA, once with 300 μ l of 80% ACN, 0.1% TFA and phosphopeptides were eluted in 180 μ l of 1% NH_4OH , pH 11, and acidified with TFA. Phosphopeptides were desalted on Oasis HLB μ -elution plates and eluted in 400 μ l of 80% ACN, 0.1% FA after which they were dried. Samples were suspended in 0.1% FA and analysed by LC-MS/MS.

LC-MS/MS analysis

Phosphopeptide samples were analysed on an EASY-nLC 1000 (mobile phase A was 0.1% FA in water and mobile phase B was 0.1% FA in ACN) connected to an Orbitrap Elite (Thermo) equipped with a nanoelectrospray ion source. For SCX fractions enriched by TiO_2 peptides were loaded on to a Dionex Acclaim[®] PepMap100 trap column [Thermo, 75 $\mu\text{m} \times 2$ cm, C18 3 μm 100 $\text{\AA}$ (1 $\text{\AA} = 0.1$ nm)] and separated on a Dionex Acclaim[®] PepMap RSLC analytical column (Thermo, 50 $\mu\text{m} \times 15$ cm, C18 2 μm 100 $\text{\AA}$) at a flow rate of 300 nl/min using a linear gradient of 2%–30% B for 60 min, 30%–98% B for 5 min, then holding at 98% for 10 min. For peptides enriched by TiO_2 without prior SCX fractionation the gradient was 2%–30% B for 120 min but all other parameters were the same. The nano-source capillary temperature was set to 275°C and the spray voltage was set to 2 kV. MS1 scans were acquired in the Orbitrap Elite at a resolution of 60000 full width half maximum (FWHM, 400–1600 m/z) with an AGC target of 1×10^6 ions over a maximum of 500 ms.

MS2 spectra were acquired for the top 15 ions from each MS1 scan in rapid scan mode in the ion trap with a target setting of 1×10^4 ions, an accumulation time of 100 ms and an isolation width of 2 Da. The normalized collision energy was set to 35% and one microscan was acquired for each spectrum. Monoisotopic precursor selection was enabled and only MS1 signals exceeding 1000 counts triggered the MS2 scans, with +1 and unassigned charge states not being selected for MS2 analysis. Dynamic exclusion was enabled with a repeat count of 2, repeat duration of 30 s and exclusion duration of 90 s.

Database searching and post processing

All raw MS/MS data were searched using the Sorcerer 2TM-SEQUEST[®] algorithm (Sage-N Research). Data were searched against the May 2014 Uniprot rat canonical database allowing for: carbamidomethyl (fixed modification), oxidation (variable modification) and phosphorylation (variable modification). Tolerances were: 1.00 Da for fragments and 50 ppm for parent ions and allowing for two missed cleavages. Analysis was done in Scaffold 4 (v4.3.4, Proteome Software) with protein and peptide probability thresholds of 95% and 90% respectively [providing a protein false discovery rate (FDR) of 0.5% and a peptide FDR of 0.4%]. The ambiguity score (Ascore) method was used to determine the confidence in correctly assigning phosphorylation sites based on the presence and intensity of site-determining ions in the MS/MS spectra [17]. Only sites with Ascores above 12, which corresponds to a localization probability of >80%, are reported. Additional analysis was done using Scaffold PTM (v2.1.3, Proteome Software). For analysis, spectral counts were summed over all fractions per sample. The MS proteomics data (phosphorylation sites detected on PKC δ as well as other cellular proteins) have been deposited to the ProteomeXchange Consortium [18] via the PRIDE partner repository with the dataset identifier PXD002536. Phosphorylation sites were manually validated and searched through phosphosite (<http://www.phosphosite.org/homeAction.do>, which curates results from phosphorylated amino acid residues observed in high throughput proteomics studies) and Uniprot (<http://www.uniprot.org/>, for other pertinent information available for known sites).

PKC δ mutants

The pPKC δ -EGFP construct (which expresses rat PKC δ with EGFP fused to its C-terminus) was generously provided by Dr Mary Reyland (University of Colorado Health Sciences Center). pPKC δ -S130D, pPKC δ -T141E, pPKC δ -S130D/T141E and pPKC δ -S130F were generated by site-directed mutagenesis according to the manual for the Quick-ChangeSite-directed Mutagenesis Kit (Stratagene). PKC δ expression plasmids were introduced into human embryonic kidney (HEK)293 cells by Effectene Transfection Reagent (Qiagen) according to the instruction manual. Cells were grown for 24 h, lysed in homogenization buffer (20 mM Tris/Cl, pH 7.5, 0.05 mM EDTA, 0.5 mM DTT, 0.2% Triton X-100, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 5 μ g/ml benzamidine, 1 mM PMSF, 5 μ M pepstatin A). Lysates either were subjected to direct immunoblot analysis or to immunoprecipitation with mouse monoclonal anti-GFP antibody 3E6 (Invitrogen) for *in vitro* kinase assays (IVKAs).

In vitro kinase assays and immunoblotting studies

IVKAs were performed with PKC δ immunoprecipitated from 150 μ g of starting cell extract according to methods published previously [4,6]. Incubations were performed for 30 min at

30°C in the absence or presence of 89 µg/ml phosphatidylserine plus 175 nM PMA in 110 µl of a reaction buffer containing 30 mM Tris/Cl, pH 7.5, 5.45 mM MgCl₂, 0.65mM EDTA, 0.65mM EGTA, 0.1 mM DTT, 1.09 mM sodium orthovanadate, 0.1 µM calyculin, 0.55 µM protein kinase inhibitor (PKI), 217 mM NaCl, 3.6% glycerol and [γ -³²P]ATP (10 µCi, 66 µM) with 4 µg of troponin complex [consisting of equimolar amounts of recombinant cardiac troponin I (cTnI), cardiac troponin T and cardiac troponin C] as the substrate. Methods to prepare the recombinant troponin protein complex have been described previously [19].

Immunoblotting was performed according to methods described previously [6] or manufacturer's instructions with antibodies from the following sources: PKC δ -pThr⁵⁰⁵, PKC δ -pSer³⁵⁷, PKC δ -pSer⁶⁴³, PKC δ -pSer⁶⁶², troponin I-pSer²³/Ser²⁴ and the anti-pTxR motif antibody were purchased from Cell Signaling Technology; antibodies that recognize PKC δ and actin were purchased from Santa Cruz Biotechnology. In each figure, each panel represents the results from a single gel (exposed for a uniform duration); detection was with ECL. ³²P incorporation was quantified by phosphorimager and PKC δ phosphorylation was normalized to the PKC δ protein level measured in the sample.

RESULTS AND DISCUSSION

MS analysis of PKC δ phosphorylation sites: the identification of a novel PKC δ phosphorylation site at Ser¹³⁰

MS analyses were performed on lysates prepared from resting and PMA-stimulated neonatal rat ventricular cardiomyocytes that heterologously overexpress PKC δ . All aspects of the procedure (the SCX fractionation, TiO₂ enrichment and LC-MS/MS analysis) were performed in parallel on the resting and PMA-treated samples. This unbiased phosphoproteomic analysis, which was performed on relatively complex whole-cell lysate samples, identified 17 phosphorylation sites on PKC δ ; almost all of the PKC δ phosphorylation sites detected in previous studies were captured in this analysis (Figure 1, the data supplement; Table 1 and references cited therein). However, the Ser³⁵⁷ phosphorylation site identified in our previous more targeted MS study ([5] which used in-gel trypsin digestion of the immunoprecipitated PKC δ band followed by a more targeted multiple reaction monitoring MS approach to specifically measure phosphorylated and unphosphorylated Ser³⁵⁷ peptide species) was not captured in the present study. The failure to detect the Ser³⁵⁷ phosphorylation site in this set of experiments could be due to differences in MS methods/instrumentation, differences in sample complexity (whole cell lysate compared with in-gel digestion of only the PKC δ band) or the different database search engines used for the analysis. It is not due to a defect in PKC δ -Ser³⁵⁷ phosphorylation, since this phosphorylation site is readily detected by immunoblot analysis on these samples (Figure 4). An analysis on cell lysates (rather than purified cellular preparations) also is skewed to detect the more abundant cellular proteins. As a result, certain myofilament proteins (such as cardiac troponins) typically are under-represented in MS studies of neonatal ventricular cardiomyocytes that have relatively immature/underdeveloped sarcomeric structures. This communication focuses on the serine and

threonine phosphorylation sites identified on PKC δ ; sites on other cellular proteins are the focus of ongoing studies.

The MS analysis identified the three known ‘priming’ phosphorylation sites at the activation loop (Thr⁵⁰⁵) and the C-terminal turn motif (Ser⁶⁴³) and the hydrophobic motif (Ser⁶⁶²). Consistent with previous evidence that C-tail phosphorylations are constitutive modifications that are not regulated during enzyme activation, C-terminal ‘priming’ phosphorylations at Ser⁶⁴³ and Ser⁶⁶² were identified in lysates from both resting and PMA-stimulated cardiomyocytes. In contrast, activation loop (Thr⁵⁰⁵) phosphorylation was detected exclusively in the PMA-treated samples. This result is consistent with previous Western blotting studies showing that PMA treatment leads to an increase in activation loop phosphorylation on the endogenous PKC δ enzyme in cardiomyocytes {although it is at odds with a similar analysis of the heterologously overexpressed enzyme which shows similar levels of constitutive PKC δ -Thr⁵⁰⁵ phosphorylation in resting and PMA-treated cardiomyocytes (Figure 3); [4]}. The MS analysis also identified two known phosphorylation sites in the hinge region at Thr²⁹⁵ and Ser²⁹⁹. These sites, which are typically used as surrogate markers of PKC δ activity, were detected exclusively in the PMA-treated samples.

The MS analysis detected phosphorylation sites at Thr²¹¹, Thr²¹³, Ser²¹⁵ and Thr²¹⁸ in the C1A–C1B interdomain. The Thr²¹⁸ phosphorylation site was identified in previous MS analyses [6,10], but phosphorylation at Thr²¹¹, Thr²¹³ and Ser²¹⁵ was not previously identified. These sites form a phosphorylation cluster (or ‘hot spot’) in the C1A–C1B interdomain region of the enzyme. Although these phosphorylations could, in theory, be constitutive modifications that have no bearing on enzyme activation, the observation that several of these phosphorylation sites were detected primarily in PMA-treated cardiomyocyte samples suggests that these phosphorylation sites might be markers (or mediators) of enzyme activation.

Three phosphorylation sites were identified in the C2 domain; the phosphorylation site at Thr⁵⁰ is listed in phosphosite (which curates results of high throughput phosphoproteomic studies), but Thr³⁷/Thr³⁸ phosphorylation has not previously been reported. Progress in understanding the functional role of PKC δ ’s C2 domain as a phosphotyrosine-binding domain (that interacts with phosphotyrosines in a (Y/F)-(S/A)-(V/I)-pY-(Q/R)-X-(Y/F) consensus binding motif [20]) provides a framework to consider the functional consequences of these phosphorylations. Specifically, structural modelling studies suggest that the highly conserved Met⁵¹/Tyr⁵² residues (adjacent to Thr⁵⁰) provide critical contacts for pTyr-peptide recognition; these residues form a hydrophobic pocket that docks the +3 position aromatic residue in the interacting pTyr-bearing protein. It is reasonable to speculate that a phosphorylation at the neighbouring Thr⁵⁰ position in the C2 domain would influence pTyr-protein binding. A framework to consider the functional consequences of Thr³⁷/Thr³⁸ phosphorylation (which is detected in the enzyme recovered from PMA-treated, but not resting cardiomyocytes) is less obvious, although introduction of a charge at these other positions might also be functionally important.

Finally, we identified PKC δ phosphorylation at Ser¹³⁰, a site that has been identified in several previous large-scale MS studies [21–24] but whose functional consequences have never been considered. Manual inspection of the spectrum (Figure 2) confirms that Ser¹³⁰ is the modified residue, as there are sufficient y- and b-series ions to unambiguously assign this site; the Ascore of 126 corresponding to this spectrum supports this claim (Table 1). We chose to examine the functional importance of Ser¹³⁰ phosphorylation for the following reasons. (1) Ser¹³⁰ is strategically positioned N-terminal to the pseudosubstrate domain (and the regulatory phosphorylation site at Thr¹⁴¹). (2) The pSer¹³⁰-bearing peptide was detected at high frequency in our studies. Whereas Ser¹³⁰-phosphorylation was detected in both resting and PMA-treated samples, it was detected almost three times more frequently in the PMA-treated samples indicating that it accompanies and/or contributes to PKC δ activation. (3) The residues flanking Ser¹³⁰ conform to a PKC consensus phosphorylation motif (a serine flanked by arginine or lysine at –2 and +2 positions), which is well suited to support an autophosphorylation reaction. (4) Ser¹³⁰ is evolutionarily conserved in PKC δ . Whereas this specific phosphorylation motif is not found in other PKC isoforms, recent efforts to profile the phosphoproteome of various human cell lines and tissues has resulted in the identification of phosphorylation sites (albeit in different motifs) at similar positions relative to the pseudosubstrate domains in all three conventional PKCs (PKC α , PKC β and PKC γ [25–28]; Figure 1). A functionally important phosphorylation site also has been identified N-terminal to the pseudosubstrate domain of PKC ζ [29] (Figure 1). This could suggest that this region of the enzyme is another phosphorylation ‘hot spot’ and that phosphorylations in this region of the enzyme (i.e. at a position predicted to interfere with pseudosubstrate domain binding to the catalytic pocket) constitute a more general mechanism to control PKC activity. (5) Ser¹³⁰ is the site of a non-synonymous single-nt polymorphism (SNP). A single base pair alteration in the coding region of PKC δ gives rise to an enzyme with a phenylalanine at this position (The National Center for Biotechnology Information dbSNP website; <http://www.ncbi.nlm.nih.gov/SNP>). A Ser130Phe substitution (which replaces the hydroxy group for an aromatic ring) would prevent phosphorylation, but it also in theory might exert an independent effect on enzyme activation (if this site was part of an extended pseudosubstrate domain-binding surface in the catalytic pocket). Therefore, we used a mutagenesis strategy to examine the functional consequences of phosphorylation or phenylalanine substitution at Ser¹³⁰.

Ser¹³⁰ regulates *in vitro* PKC δ activity

Since preliminary studies indicated that phosphomimetic or serine→phenylalanine substitutions at position 130 or a phosphomimetic substitution at position 141 (adjacent to the pseudosubstrate domain) do not grossly alter PKC δ expression or *in vivo* ‘priming’ site (Thr⁵⁰⁵, Ser⁶⁴³, Ser⁶⁶²) phosphorylation (Figure 3A), we used IVKA methods to determine whether these modifications influence PKC δ activity. This approach (which is described and validated in our previous publications [4,6]) involved the immunoprecipitation of heterologously overexpressed WT and mutant enzymes from HEK293 cells followed by IVKAs to track autocatalytic activity and activity toward a heterologous protein substrate. Autocatalytic activity is tracked by immunoblot analysis with an anti-pTxR motif phosphorylation-site-specific antibody (PSSA) that specifically detects PKC δ autophosphorylation at Thr²⁹⁵ [6]. Activity toward serine and threonine P-sites on a

heterologous protein substrate is assessed by tracking phosphorylation of cTnI (a thin filament protein that is a physiologically important substrate for PKC δ in cardiomyocytes). cTnI contains a serine phosphorylation cluster at Ser²³/Ser²⁴ (which is recognized by the anti-cTnI-pSer²³/Ser²⁴ PSSA) as well as a threonine phosphorylation site at Thr¹⁴⁴ (which is flanked by a +2 position arginine and therefore is recognized by the anti-TxR motif PSSA). Although stimulus-dependent changes in cTnI phosphorylation at these sites have been implicated as a physiologically relevant mechanism that dynamically regulates cardiac mechanics, cTnI phosphorylation is monitored in the present study simply as a convenient strategy to simultaneously track and discriminate PKC δ 's serine compared with threonine kinase activities.

Figure 3(B) shows that WT-PKC δ is a strictly lipid-dependent enzyme. WT-PKC δ autophosphorylates at Thr²⁹⁵ and it phosphorylates cTnI at Ser²³/Ser²⁴ only following activation by PS/PMA. Of note, the allosterically-activated WT-PKC δ enzyme is a serine kinase that does not phosphorylate cTnI at Thr¹⁴⁴. In contrast, PKC δ -S130 D and PKC δ -T141E constructs display considerable amounts of basal Thr²⁹⁵ autocatalytic activity and a modest level of basal lipid-independent activity toward cTnI; PKC δ -S130D and PKC δ -T141E activities are further increased by PS/PMA. This is detected by immunoblot analysis with antibodies that track PKC δ activity toward individual Psites on cTnI (Figure 3B) and quantified based upon ³²Pincorporation, which provides an integrated measure of multisite cTnI phosphorylation at Ser²³/Ser²⁴ and Thr¹⁴⁴ (Figure 3B). Importantly, the Western blotting studies show that WT-PKC δ specifically phosphorylates cTnI at Ser²³/Ser²⁴ (not Thr¹⁴⁴), but PKC δ -S130D and PKC δ -T141E do not discriminate between these sites; they phosphorylate cTnI at both Ser²³/Ser²⁴ and Thr¹⁴⁴. Finally, Figure 3 shows that the PKC δ -S130D/T141E double mutant is a constitutively active enzyme. PKC δ -S130D/T141E displays high levels of serine and threonine kinase activity that are not further increased by PS/PMA. These results implicate phosphorylations at sites adjacent to the pseudosubstrate domain as post-translational modifications that disrupt auto-inhibitory constraints that limit basal catalytic activity and influence substrate (P-site) specificity.

Additional studies showed that a non-phosphorylatable S130A substitution does not induce any gross changes in PKC δ kinase activity (results not shown), but a S130F substitution that mimics the SNP identified in some human populations induces a modest increase in PKC δ 's maximal lipid-dependent catalytic activity (Figure 3B). Of note, the S130F substitution also alters PKC δ 's substrate specificity. Figure 3(B) shows that WT-PKC δ phosphorylates cTnI at Ser²³/Ser²⁴ (not Thr¹⁴¹), but PKC δ -S130F is a serine/threonine kinase that phosphorylates cTnI at both Ser²³/Ser²⁴ and Thr¹⁴⁴ (much like PKC δ -S130D). Whereas PKC δ -S130F displayed a low level of basal lipid-independent activity in some experiments, this effect of the S130F substitution was neither consistent nor statistically significant. The observation that a S130F substitution (which mimics a SNP identified in certain human populations) increases PKC δ 's maximal lipid-dependent catalytic activity and also confers threonine kinase activity may have relevance to various PKC δ -driven clinical disorders (such as ischaemia-reperfusion injury and certain malignancies). It is worth noting that two other polymorphic variations have been mapped to a neighbouring site in the enzyme. R132C or R132H substitutions (that replace an arginine residue at the +2 position relative to the Ser¹³⁰ phosphorylation site with either cysteine or histidine residues) have been identified in certain

human populations (each with aMAF of 0.002). These SNPs that disrupt the PKC consensus phosphorylation motif are predicted to decrease Ser¹³⁰ phosphorylation and also might lead to a clinical phenotype.

Ser¹³⁰ and Thr¹⁴¹ phosphorylation sites control PKC δ G loop phosphorylation at Ser³⁵⁷

We recently showed that PKC δ is recovered from cardiomyocytes as a G loop Ser³⁵⁷-phosphorylated enzyme and that Ser³⁵⁷ phosphorylation limits PKC δ 's threonine kinase activity [5]. We used an immunoblotting approach with a PKC δ -pSer³⁵⁷ PSSA to determine whether the S130D and T141E substitutions increase PKC δ 's threonine kinase activity indirectly by decreasing G loop phosphorylation at Ser³⁵⁷. Figure 4 shows that WTPKC δ is detected as a Ser³⁵⁷ phosphorylated enzyme in resting HEK293 cells. Whereas the stoichiometry of PKC δ -Ser³⁵⁷ phosphorylation was not quantified in the previous study performed in cardiomyocytes [5], Figure 4 shows that WT-PKC δ -Ser³⁵⁷ phosphorylation increases in response to treatment of HEK293 cells with PMA, indicating that HEK293 cells must contain a pool of enzyme that lacks Ser³⁵⁷ phosphorylation. The PMA-dependent increase in WT-PKC δ -Ser³⁵⁷ phosphorylation is prevented by the PKC inhibitor GF109203X (Figure 4B), indicating that this is mediated by a PKC-dependent (possibly autocatalytic) reaction.

Figure 4(A) shows that S130D and T141E substitutions lead to a profound Ser³⁵⁷ phosphorylation defect; PKC δ -S130D and PKC δ -T141E display little-to-no basal Ser³⁵⁷ phosphorylation and a markedly attenuated PMA-dependent Ser³⁵⁷ phosphorylation response. Conversely, the S130A substitution tended to exaggerate the PMA-dependent Ser³⁵⁷ phosphorylation response (Figure 4C). Control experiments show that the inhibitory effects of the S130D and T141E substitutions cannot be attributed to any gross structural abnormality that prevents ATP positioning loop phosphorylation at Ser³⁵⁷, since PKC δ -S130D, PKC δ -T141E and PKC-S130D/T141E mutants show high levels of Ser³⁵⁷ phosphorylation (similar to WT-PKC δ) when cells are treated with the phosphatase inhibitor calyculin A (result not shown). These results suggest that the Ser³⁵⁷ phosphorylation site is subject to dual control by cellular kinases and phosphatases and that an auto-inhibitory interaction between the pseudosubstrate domain and the active site protects Ser³⁵⁷ from dephosphorylation, whereas phosphorylation at sites adjacent to the pseudosubstrate domain (Ser¹³⁰ and/or Thr¹⁴¹) displaces the pseudosubstrate domain from the active site and favours Ser³⁵⁷ dephosphorylation. The Ser³⁵⁷ phosphorylation defect that results from the S130D/T141E substitutions is noteworthy in the context of our previous studies that implicated a redox-dependent tyrosine phosphorylation at position 311 as a mechanism that controls G loop phosphorylation at Ser³⁵⁷. The observation that Ser¹³⁰/Thr¹⁴¹ phosphorylation constitutes an alternative mechanism to regulate G loop phosphorylation (in the absence of any changes in tyrosine phosphorylation) suggests that PKC δ is equipped with at least two mechanisms to regulate its threonine-kinase activity.

Finally, Figure 4(A) also shows that activating phosphorylations adjacent to the pseudosubstrate domain (that prevent inhibitory G loop phosphorylation at Ser³⁵⁷) have a profound effect on maximal PKC δ activity in a cellular context. Specifically, PMA induces a modest increase in WT-PKC δ -Thr²⁹⁵ phosphorylation; the PMA-dependent Thr²⁹⁵

phosphorylation response is markedly exaggerated in cells expressing the PKC δ mutants harbouring S130D or T141E substitutions. In each case, the PMA-dependent changes in Thr²⁹⁵ phosphorylation are not associated with detectable changes in enzyme phosphorylation at priming sites in the activation loop and C-terminus.

Ser¹³⁰ and Thr¹⁴¹ phosphorylations influence PKC δ down-regulation in cells

Previous studies implicated Thr¹⁴¹ autophosphorylation as a post-translational modification that controls PKC δ activation. Since Ser¹³⁰ maps to a similar region of the enzyme, we examined whether this residue also contributes to the regulation of PKC δ down-regulation (a convenient marker of enzyme activation in cells). Figure 5 shows that WT-PKC δ expression levels remain relatively constant in HEK293 cells treated for 24 h with vehicle, but PMA treatment leads to the down-regulation of S130D-, T141E-, S130D/T141E- and (to a lesser extent) S130F-substituted forms of PKC δ . These results indicate that a negative charge (or a bulky substitution) in the vicinity of the PKC δ pseudosubstrate domain at Ser¹³⁰ and/or Thr¹⁴¹ favours PMA-dependent down-regulation.

CONCLUSION

Our previous studies identified Thr¹⁴¹ as an autophosphorylation site adjacent to the pseudosubstrate domain that regulates PKC δ activation. The present study identifies a second phosphorylation site in this region of the enzyme at Ser¹³⁰. Although our experiments did not examine the mechanism for Ser¹³⁰ phosphorylation, it is interesting to note that Ser¹³⁰ is flanked by a consensus PKC phosphorylation motif. Although this could suggest that Ser¹³⁰ might also be a target for an autocatalytic reaction, a *trans*-phosphorylation by some other kinase *in vivo* in a cellular context also is possible (and these mechanisms are not mutually exclusive). In either case, our results implicate phosphorylation sites at Ser¹³⁰ and Thr¹⁴¹ (adjacent to the pseudosubstrate domain) as post-translational modifications that co-operate with the G loop phosphorylation site to regulate PKC δ activity and substrate specificity.

Our results shine a spotlight on the pseudosubstrate domain as a region of the enzyme that plays a more complex and heretofore unrecognized role in the activation mechanism for PKC δ and possibly other PKCs. Specifically, the conventional model holds that the pseudosubstrate domain engages in intramolecular autoinhibitory interactions with the active site that maintain low basal levels of PKC activity; the conformational transition that accompanies enzyme activation relieves pseudosubstrate domain-mediated auto-inhibition and leads to enzyme activation. The notion that phosphorylation provides an additional mechanism to expel the pseudosubstrate domain from the active site and promote PKC activation has never been considered. However, autophosphorylations adjacent to pseudosubstrate domain autoinhibitory sequences are a characteristic feature of the activation mechanism for calmodulin-dependent protein kinase family enzymes [30–32], functionally important phosphorylation sites flank the pseudosubstrate domain of the cAMP-dependent protein kinase type I- α regulatory chain [33–35] and a phosphorylation site has been mapped to a position adjacent to the auto-inhibitory pseudosubstrate domain of p21-activated kinase (PAK4) [36]. The observation that the pseudosubstrate domain region also

is a phosphorylation ‘hot spot’ in multiple PKC isoforms (Figure 1) suggests that dynamically-regulated phosphorylations may be a more general feature of activation mechanisms for cellular enzymes such as PKCs. Finally, it is worth noting that pseudosubstrate domain sequences are required for the proper *in vivo* translocation and activation of several PKCs (including PKC β , PKC ϵ , PKC η and PKC ζ [37–41]); in some cases, this has been attributed to specific inter-molecular interactions between basic residues in the pseudosubstrate domain and acidic-binding surfaces on targeting proteins [37,38]. These newer results lend further support to the notion that molecular determinants in the pseudosubstrate domain region that actively contribute to the activation process could be targeted for therapeutic advantage.

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Abbreviations

ACN	acetonitrile
cTnI	cardiac troponin I
DAG	diacylglycerol
FA	formic acid
HEK	human embryonic kidney
HLB	hydrophilic lipophilic balanced
IVKA	<i>in vitro</i> kinase assay
PKC	protein kinase C
P-site	phosphoacceptor site
PKD	protein kinase D
PS	phosphatidylserine
PSSA	phosphorylation-site-specific antibody
SCX	strong cation exchange
SNP	single-nt polymorphism
TFA	trifluoroacetic acid
WT	wild-type

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Figure 1. Schematic that maps phosphorylation sites to different structural domains in PKCδ
 Top: Domain structure of PKCδ with the conserved C2-like and C1A–C1B regions in the regulatory domain and the kinase domain in the catalytic domain depicted in blue. The human (h), rat (r) and mouse (m) sequences that flank each phosphorylation site identified in the present study are provided to show that these phosphorylation motifs are evolutionarily conserved. The known priming phosphorylation sites are depicted in green, the sites identified in our previous study are depicted in blue [7] and the sites newly identified in this study are depicted in red. Bottom: Sequences flanking the pseudosubstrate domains of PKCδ (RRGAIK) and cPKCs (RKGALR) are aligned to show that phosphorylation sites (depicted in red) map to similar positions in these enzyme.

Figure 2. MS2 spectrum for pSer¹³⁰ peptide (VLMcVQYFLEDGDcKQSPHOMR) with precursor m/z of 820.3423 (+3 charge)

Sequence with b and y ion series is shown with fragment ions (or any variation thereof) identified highlighted in red. The site of phosphorylation at Ser¹³⁰ is indicated and carbamidomethyl cysteines are indicated with a lower case.

Figure 4. Phosphomimetic substitutions at Ser¹³⁰ and Thr¹⁴¹ disrupt G loop phosphorylation at Ser³⁵⁷

(A and C) HEK293 cells that heterologously overexpress WT-PKCδ or the indicated Ser¹³⁰ and/or Thr¹⁴¹ substituted PKCδ mutants were treated with vehicle or 200 nM PMA for 20 min and then subjected to immunoblot analysis for PKCδ or PKCδ phosphorylation. The Western blots are representative of three experiments performed on separate culture preparations. PMA-dependent increases in Ser³⁵⁷ immunoreactivity were quantified (with PKCδ immunoreactivity serving as protein loading control). Values were normalized to the level of Ser³⁵⁷ phosphorylation in PS/PMA-treated WT-PKCδ samples and show that Ser³⁵⁷ phosphorylation is significantly impaired in the PKCδ-S130D mutant ($n = 3$, $P < 0.05$). Ser³⁵⁷ phosphorylation tended to be higher in the PKCδ-S130A construct compared with WT-PKCδ, but this difference did not reach statistical significance. (B) HEK293 cells that heterologously overexpress WT-PKCδ were pre-treated for 45 min with vehicle or 10 μ M GF019023X (GFX) followed by stimulation with vehicle or 200 nM PMA for 20 min.

Figure 5. Ser¹³⁰ and Thr¹⁴¹ co-operate to regulate PKCδ down-regulation in HEK293 cells
 HEK293 cells that heterologously overexpress WT-PKCδ, PKCδ-S130D, PKCδ -T141E, PKCδ-S130D/T141E or PKCδ-S130F were treated with vehicle or 200 nM PMA for 24 h. A representative experiment depicting PMA-dependent changes in PKCδ protein immunoreactivity for each construct is depicted in the left panel, with the PMA-dependent change in PKCδ immunoreactivity for each construct (normalized against actin loading controls) quantified on the right (n =5, *P <0.05 compared with WT-PKCδ).

Table 1

Summary of the phosphopeptides derived from PKC δ detected by Orbitrap MS of PKC δ isolated from resting or PMA-stimulated cardiomyocytes.

Site	Flanking sequence	Observed peptide	Best Score*	Localization probability	Basal	PMA	Previously reported	Location in enzyme	Deviation from human
Thr ³⁷	VKMKEALITDRGKTLV	MIKEALITDRGK	1000.00	100%	N	Y	No		Serine in human
Thr ³⁸	VKMKEALITDRGKTLV	MKEALITDRGK	1000.00	100%	N	Y	No	C2 Domain	
Thr ⁵⁰	TLVOKKPTIWPPEWKS	KPIIWPPEWK	55.92	100%	Y	Y	1		
Ser ¹³⁰	EDGDKCKSMRSEEEA	VLMcVQYFLEDGDcKQsMR	126.05	100%	Y	Y	1	Pseudosubstrate domain	
Thr ¹⁶⁴	KNHEFIATFFGQPTI	NHEFIATFFGQPTFcSVcK	70.66	100%	N	Y	No	C1 domain	
Thr ²¹¹	IIGRCTGTAINSRDT	cTGTATNsRDTIFQK	13.34	95%	N	Y	No	C1A/C1B interdomain	Alanine in human
Thr ²¹³	GRCGTGTAINSRDTIF	cTGTATNsRDTIFQK	12.5	89%	Y	Y	No		
Ser ²¹⁵	CTGTATNSRDTIFQK	cTGTATNsRDTIFQK	12.81	86%	N	Y	No		
Thr ²¹⁸	TATNSRDTIFQKERF	cTGTATNSRDTIFQK	25.67	100%	N	Y	1, 2		
Thr ²⁹⁵	AEALNQVSKASRKP	LLAEALNQVQK	1000.00	100%	N	Y	2	Hinge	
Ser ²⁹⁹	NQVTOKASRKPEPTE	LLAEALNQV'TQKAsR	1000.00	100%	N	Y	1, 3		
Ser ⁵⁰⁴	IFGENRASTFCGTPD	AsIFcGTPDYIAPEILQGLK	32.12	100%	N	Y	1, 2	Activation loop	
Thr ⁵⁰⁵	FGENPASTFCGTPDY	AsIFcGTPDYIAPEILQGLK	33.81	100%	N	Y	1, 2		
Ser ⁶⁴³	LINEKPOLSFSDKNLI	VKSPSDY'SNFDPEFLNEKPQLsFS DK	22.45	100%	Y	Y	1, 2	V5-turn motif	
Ser ⁶⁴⁵	EKPQLSFSDKNLIDS	VKSPSDY'SNFDPEFLNEKPQLsFS DK	14.63	97%	Y	Y	1	V5 variable region	
Thr ⁶⁵⁶	LIDSMDDQATFKGFSF	NLIDSMDDQAFKGFsFV'NPK	18.19	99%	N	Y	1	V5 variable region	Serine in human

Site	Flanking sequence	Observed peptide	Best Ascore [*]	Localization probability	Basal	PMA	Previously reported	Location in enzyme	Deviation from human
Set ⁶⁶²	QTAFKGF ^S FVNPKE	GFsFV/NPK	1000.00	100%	Y	Y	1, 2	V5-hydrophobic motif	

^{*} Ascore reflects the confidence in localization of the modification to the assigned residue.
1 Reported in PhosphoSite. 2: Reported in Rybin et al. [6]; 3: Reported in Durgan et al. [7].
Abbreviations: N, no; Y, yes.