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REGULATORY AUTOPHOSPHORYLATION SITES ON PROTEIN KINASE C- δ AT Thr¹⁴¹ AND Thr²⁹⁵

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Abstract

Protein kinase C- δ (PKC δ) is a Ser/Thr kinase that regulates a wide range of cellular responses. This study identifies novel *in vitro* PKC δ autophosphorylation sites at Thr¹⁴¹ adjacent to the pseudosubstrate domain, Thr²¹⁸ in the C1A–C1B interdomain, Ser²⁹⁵, Ser³⁰², and Ser³⁰⁴ in the hinge region, and Ser⁵⁰³ adjacent to Thr⁵⁰⁵ in the activation loop. Cell-based studies show that Thr¹⁴¹ and Thr²⁹⁵ also are phosphorylated *in vivo* and that Thr¹⁴¹ phosphorylation regulates the kinetics of PKC δ downregulation in COS7 cells. *In vitro* studies implicate Thr¹⁴¹ and Thr²⁹⁵ autophosphorylation as modifications that regulate PKC δ activity. A T141D substitution markedly increases basal lipid-independent PKC δ activity; the PKC δ -T141D mutant is only slightly further stimulated *in vitro* by PMA treatment, suggesting that Thr¹⁴¹ phosphorylation relieves autoinhibitory constraints that limit PKC δ activity. Mutagenesis studies also indicate that a phosphorylation at Thr²⁹⁵ contributes to the control of PKC δ substrate specificity. We previously demonstrated that PKC δ phosphorylates the myofilament protein cardiac troponin I (cTnI) at Ser²³/Ser²⁴ when it is allosterically activated by lipid cofactors and that the Thr⁵⁰⁵/Tyr³¹¹-phosphorylated form of PKC δ (that is present in assays with Src) acquires as additional activity toward cTnI-Thr¹⁴⁴. Studies reported herein show that a T505A substitution reduces PKC δ -Thr²⁹⁵ autophosphorylation and that a T295A substitution leads to a defect in Src-dependent PKC δ -Tyr³¹¹ phosphorylation and PKC δ -dependent cTnI-Thr¹⁴⁴ phosphorylation. These results implicate PKC δ -Thr²⁹⁵ autophosphorylation as a lipid-dependent modification that links PKC δ -Thr⁵⁰⁵ phosphorylation to Src-dependent regulation of PKC δ catalytic function. Collectively, these studies identify novel regulatory autophosphorylations on PKC δ that serve as markers and regulators of PKC δ activity.

Keywords

protein kinase C-delta; autophosphorylation; enzyme activity; Src; cardiac troponin I

Protein kinase C δ (PKC δ) is a member of the PKC family of serine/threonine kinases that sit at the crossroads of signal transduction pathways implicated in a wide range of cellular responses (1). PKC δ 's structure consists of a highly conserved C-terminal catalytic domain (consisting of motifs required for ATP/substrate-binding and catalysis) and an N-terminal regulatory domain containing tandem C1A–C1B domains that bind lipid cofactors, a C2 domain that acts as a protein-protein interaction module, and an autoinhibitory pseudosubstrate domain (a sequence lacking a serine/threonine phosphoacceptor site, but otherwise resembling a PKC substrate) that maintains the enzyme in an inactive conformation. Like other PKC isoforms, PKC δ is activated by growth factor receptors that promote the accumulation of lipid cofactors such as diacylglycerol (DAG) or by tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate [PMA]) that anchor the enzyme in an active conformation to

membranes. However, recent studies expose phosphorylation at a highly-conserved threonine residue in the activation loop (Thr⁵⁰⁵) as an additional component of the PKC δ activation mechanism (2). For most PKC isoforms, activation loop phosphorylation is a stable ‘priming’ modification that is mediated by PDK-1 and serves (along with additional phosphorylation events at conserved turn and hydrophobic motifs in the C-terminus) to lock the enzyme in a mature catalytically-competent conformation, poised to be allosterically activated by lipid cofactors (3). In contrast, PKC δ is catalytically active even without Thr⁵⁰⁵ phosphorylation. Rather, PKC δ -Thr⁵⁰⁵ phosphorylation has emerged as a dynamically regulated autocatalytic reaction that ‘fine-tunes’ PKC δ ’s catalytic function toward selected substrates (4).

While *in vitro* intramolecular autophosphorylations of brain PKC were described over 20 years ago (5), conventional models of PKC isoform activation do not consider a possible role for autophosphorylation reactions that regulate PKC enzyme activity. Nevertheless, an early study mapped PKC- β II autophosphorylation to Ser¹⁶ and Thr¹⁷ in the N-terminus, Thr³¹⁴ and Thr³²⁴ in the hinge region, and Thr⁶⁴¹ in the C-terminus (6). Thr⁶⁴¹ resides in the turn motif, a highly conserved phosphorylation motif in all PKC isoforms. Other PKC β II autophosphorylation sites in the hinge region and N-terminus are not conserved in other PKC isoforms; since these autophosphorylation sites were not identified in subsequent studies that mapped *in vivo* PKC β II phosphorylation to Thr⁵⁰⁰ (the activation loop), Thr⁶⁴¹ (the turn motif) and Ser⁶⁶⁰ (the hydrophobic motif (7)), N-terminal and hinge region phosphorylation sites in PKCs were largely ignored in subsequent literature. The single exception is a modeling study from the Newton laboratory which considered a possible role for PKC β II-Ser¹⁶/Thr¹⁷ autophosphorylation as a mechanism that favors activation by lowering pseudosubstrate domain binding affinity for the catalytic pocket (6;8). Interest in PKC autophosphorylation has recently been rekindled by studies from the Parker laboratory showing that human PKC δ and PKC ϵ autophosphorylate at sites in their variable hinge regions (9;10); autophosphorylation sites in the hinge region of human PKC δ were mapped to Ser²⁹⁹, Ser³⁰², and Ser³⁰⁴ (Fig 1). PKC δ and PKC ϵ autophosphorylation reactions have generally been viewed as markers of enzyme activation. The notion that hinge region autophosphorylation reactions might regulate the catalytic properties of PKC δ has never been considered.

There is recent evidence that PKC δ activity also is controlled through tyrosine phosphorylation. As many as 8 highly conserved tyrosine residues in various regions of PKC δ ’s structure have been implicated as targets for regulatory phosphorylations. Our recent studies focused on PKC δ phosphorylation at Tyr³¹¹ and Tyr³³², residues unique to the hinge region of PKC δ that are phosphorylated *in vitro* by Src or related Src family kinases (2;4;11). We showed that PKC δ -Tyr³¹¹ phosphorylation increases *in vivo* in PMA- and H₂O₂-treated cardiomyocytes (2). While PKC δ phosphorylation at tyrosine residues and at the activation loop (at Thr⁵⁰⁵) generally are viewed as independently regulated events, our recent experiments exposed a novel form of cross-regulation, showing that Src and PKC δ -Tyr³¹¹/Tyr³³² phosphorylation enhances PKC δ -Thr⁵⁰⁵ autophosphorylation (2). We also demonstrated that phosphorylations at Tyr³¹¹ and Thr⁵⁰⁵ cooperate to ‘fine-tune’ PKC δ ’s enzymology towards cardiac troponin I (cTnI), a physiologically important PKC δ substrate in the heart (4). cTnI is the “inhibitory” subunit of the troponin complex that is critical for Ca²⁺-dependent regulation of myofilament function (12). cTnI contains three phosphorylation clusters (Ser²³/Ser²⁴, Ser⁴³/Ser⁴⁵, and Thr¹⁴⁴) that exert distinct effects on cardiac function. We showed that (1) allosterically-activated PKC δ (in assays with PS/PMA) phosphorylates cTnI at Ser²³/Ser²⁴ and that this modification leads to depressed tension at sub-maximum but not maximum [Ca²⁺] in detergent-extracted single cardiomyocytes, (2) PKC δ becomes a cTnI-Thr¹⁴⁴ kinase (and phosphorylates cTnI at both Ser²³/Ser²⁴ and Thr¹⁴⁴) and that PKC δ depresses maximum tension and cross-bridge kinetics in single detergent-extracted cardiomyocytes when it is tyrosine phosphorylated by Src, and (3) the effect of Src to convert PKC δ into a cTnI-Thr¹⁴⁴ kinase is abrogated by PKC δ -Y311F or T505A substitutions (4). These results implicate PKC δ -Tyr³¹¹/Thr⁵⁰⁵

phosphorylation as dynamically-regulated modifications that alter PKC δ enzymology and allow for stimulus-specific control of cardiac mechanics during growth factor stimulation and oxidative stress. Studies reported herein identify additional novel PKC δ autophosphorylation sites that regulate PKC δ activity.

EXPERIMENTAL

Materials

Antibodies were from the following sources: PKC δ -Thr(P)⁵⁰⁵, PKC δ -Tyr(P)³¹¹, Troponin I-Ser(P)²³/Ser(P)²⁴ and anti-pTXR, Cell Signaling Technology; PKC δ , Santa Cruz Biotechnology; mouse monoclonal anti-GFP antibody 3E6, Invitrogen. Recombinant human PKC δ was from Calbiochem. The human recombinant full-length His-tagged Src enzyme, expressed in Sf9 cells and purified by sequential chromatography (purity >95% by Coomassie blue staining of the SDS-PAGE gel) was obtained from Invitrogen. Previous studies further validated the purity of this enzyme preparation, showing that [1] the kinase activity in this preparation is completely blocked by the Src inhibitor PP1 and [2] this enzyme preparation is detected a single radioactive band corresponding to the autophosphorylated Src protein when it is subjected to *in vitro* kinase assays with ³²P-ATP and then run on a gel (i.e., no other enzymes/substrates are detected even with long exposures of the gel (4)). PMA was from Sigma. 1,2-Dioleoyl-*sn*-glycerol was from Avanti Polar Lipids, Inc. Other chemicals were reagent grade.

Cardiomyocyte culture

Cardiomyocytes were isolated from hearts of 2 day-old Wistar rats by a trypsin dispersion procedure that uses a differential attachment procedure followed by irradiation to enrich for cardiomyocytes (11). Cells were plated on protamine sulfate-coated culture dishes at a density of 5×10^6 cells/100-mm dish and grown in MEM (Gibco, BRL) supplemented with 10% fetal calf serum for 4 days and then serum-deprived for 24 hr prior to experiments.

For studies of PKC δ phosphorylation *in vivo* in cardiomyocyte cultures, cardiomyocytes were infected with an adenoviral construct that drives expression of mouse PKC δ . 2 days later, the cultures were incubated for 4–5 h at 37°C in phosphate-free MEM containing [³²P] orthophosphate (0.15–0.26 mCi/ml; 4 ml per dish). Cardiomyocytes were then treated for 10 min with vehicle or 5 mM H₂O₂ and experiments were terminated by aspirating the radioactive medium and rinsing culture dishes with a buffer containing 133 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 16.5 mM dextrose, and 20 mM HEPES. Cellular proteins were harvested in homogenization buffer (20 mM Tris-Cl, pH7.5, 0.05 mM EDTA, 0.5 mM DTT, 0.2% Triton X-100, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml benzamidine, 1 mM PMSF, 5 µM pepstatin A) and PKC δ was immunoprecipitated, separated from other cellular proteins by SDS-PAGE, and subjected to phosphopeptide mapping analysis.

PKC δ mutants

pPKC δ -EGFP (pGFP-PKC δ) was obtained as a generous gift from Dr. Mary Reyland (University of Colorado Health Sciences Center, Denver). The pPKC δ -EGFP construct expresses mouse PKC δ with enhanced GFP fused to its C-terminus. pPKC δ T141A-EGFP, pPKC δ T141D-EGFP, and pPKC δ T295A-EGFP 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 COS-7 cells by Effectene Transfection Reagent (Qiagen) according to the instruction manual. Cells were grown for 24 hours, lysed in homogenization buffer. Cell extracts were subjected to immunoprecipitation with mouse monoclonal anti-GFP antibody 3E6 (Invitrogen).

In vitro kinase assays with recombinant enzymes

In vitro kinase assays were performed with 0.032 units (32.4 ng) of recombinant human PKC δ (Calbiochem) for assays of cTn complex phosphorylation or 0.4 units for peptide mapping studies. Assays were performed in 80 μ l of a reaction buffer containing 30 mM Tris-Cl, pH 7.5, 5 mM MgCl₂, 10 mM MnCl₂, 0.9 mM EDTA, 0.9 mM EGTA, 3 mM DTT, 0.1 mM sodium vanadate, 76 mM NaCl, 23.5% glycerol, 0.006% Brij-35, 0.023% Triton X-100, 0.04 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, 83 μ g/ml phosphatidylserine (PS), 175 nM PMA, 4 μ g of troponin complex, and [γ -³²P]ATP (25 μ Ci, 97 μ M, unless indicated otherwise). Incubations were for 30 min at 30°C in the absence or presence of Src (0.66 units) or lipid cofactors. PS/PMA is included in assays to allosterically activate PKC δ and render it a better substrate for Src-dependent tyrosine phosphorylation (11).

Immunoblotting studies

Immunoblotting was performed on cell extracts according to methods described previously or manufacturer's instructions (11). In each figure, each panel represents the results from a single gel (exposed for a uniform duration); detection was with enhanced chemiluminescence and quantification was by laser scanning densitometry. All results were replicated in at least 3 experiments.

Peptide mapping studies

For peptide mapping studies, kinase reactions (see above) were stopped by adding 27 μ l of 4X SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and the band corresponding to PKC δ was excised from the membrane, cut into small pieces, and treated for 30 min at 37°C with polyvinylpyrrolidone (0.5%, w/v) in acetic acid (100 mM), followed by 5 water washes (to remove the acid) and a 10 min incubation at room temperature in the dark with 100 mM iodoacetate to carboxymethylate PKC δ . Membrane pieces were then washed three times with water and twice with 50 mM ammonium bicarbonate and incubated overnight at 37°C in 60 μ l of a buffer containing 42 mM ammonium bicarbonate, 17 μ M HCl, and 10 μ g sequencing grade trypsin. Digested peptides were eluted from the membrane by sonication, lyophilized, and the residue was reconstituted in 0.1% trifluoroacetic acid and fractionated by RP-HPLC on a Vydac semimicro C₁₈ column (2.1 $\times$ 250 mm). Peptides were eluted with a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile over 140 min at a flow rate of 1 ml/min. The eluant was monitored at 220 nm and fractions were collected every 30 sec for Cherenkov counting. Fractions containing radioactivity were subjected to LC-MS/MS analysis on a Micromass Q-ToF hybrid quadrupole/time-of-flight mass spectrometer with a nanoelectrospray source. Capillary voltage was set at 1.8kV and cone voltage 32V; collision energy was set according to mass and charge of the ion, from 14eV to 50eV. Chromatography was performed on an LC Packings HPLC with a C18 PepMap column using a linear acetonitrile gradient with flow rate of 200 nl/min. Raw data files were processed using the MassLynx version 4.0 ProteinLynx software with the MaxEnt 3 algorithm. Phosphorylated peptides were verified by manual inspection of MS/MS spectra.

RESULTS

Phospho-peptide mapping studies identify PKC δ autophosphorylation at Thr¹⁴¹, Thr²¹⁸, Thr²⁹⁵, Ser³⁰², Ser³⁰⁴ and Ser⁵⁰³

Recombinant PKC δ (human sequence) was phosphorylated *in vitro* in buffers containing [γ -³²P]ATP and PS/PMA. Since these studies were performed as part of an ongoing effort to identify sites for PKC δ phosphorylation by Src, assays were performed without and with active Src. Radiolabeled PKC δ from the *in vitro* kinase assays was purified by SDS-PAGE, blotted to nitrocellulose, excised from the membrane, and then subjected to digestion with trypsin and

fractionation by reverse phase-HPLC. Figure 2 shows that RP-HPLC chromatograms of phospho-peptide fragments derived from *in vitro* kinase assays with PKC δ , Src, and [γ - 32 P] ATP contained two distinct radioactive peaks that are not present in the chromatograms from kinase assays with PKC δ alone (Peaks 1 and 4). Sequencing of peptides in these peaks by electrospray ionization (ESI)-LC/MS/MS analysis identified phospho-Tyr 313 - and phospho-Tyr 334 -containing fragments (corresponding to phospho-Tyr 311 and phospho-Tyr 332 in rodent PKC δ , see Figure 1). Peptides containing Tyr 64 and Tyr 52 also were recovered in peak 4; while these tyrosine residues also have been identified as sites for regulatory phosphorylation, these peptide fragments were not phosphorylated. Sequencing of these and other peaks by ESI-LC/MS/MS analysis achieved ~40% coverage of the PKC δ sequence and identified six novel sites for PKC δ autophosphorylation: (1) Peak 2 contains a peptide fragment that is phosphorylated at Thr 218 ; this residue resides in a phosphorylation motif that is evolutionarily conserved in the C1A–C1B interdomain regions of PKC δ and PKC θ , but not other PKC isoforms (13). (2) Peak 3 contains a peptide fragment that is phosphorylated at Thr 141 . The Thr 141 phosphorylation motif is conserved in PKC η , but not PKC θ or other PKC isoforms. Of note, Thr 141 is positioned N-terminal to the pseudosubstrate domain of PKC δ , similar to the position of Ser 16 Thr 17 relative to the pseudosubstrate domain ($_{22}$ RKGALR 27) of PKC β II. These results suggest that PKC δ -Thr 141 and PKC β II-Ser 16 Thr 17 autophosphorylation reactions might subserve similar regulatory functions. (3) Peak 4 contains a peptide fragment that is phosphorylated at Ser 503 . Ser 503 is adjacent to the activation loop phosphorylation site in the kinase domain of human PKC δ (NIFGES 503 RAST 507); its significance is uncertain, since it is not conserved in mouse PKC δ (NIFGEG 501 RAST 505) or rat PKC δ (NIFGEN 501 RAST 505). (4) Peak 5 contains peptide fragments phosphorylated at three hinge region residues, Thr 295 , Ser 302 and Ser 304 .

Table 1 provides a summary of the PKC δ autophosphorylation sites detected in these experiments. Several aspects of the results deserve comment. First, the S 302 DS 304 ASSEPVGIY 313 QGFEK peptide fragment was recovered in three separate phospho-peptide mapping experiments as a serine-phosphorylated peptide in peak 5 or as a tyrosine-phosphorylated peptide in peak 4. In each case, the monophosphorylated peptide was detected; we never recovered a dually Ser/Tyr phosphorylated peptide. We also never recovered a dually Ser 302 /Ser 304 -phosphorylated peptide. Second, we detected PKC δ phosphorylation at the turn motif (Ser 645), but the trypsin digest failed to yield peptides containing either the activation loop (Thr 505) or hydrophobic motif (Ser 662) phosphorylation sites. While these phosphorylation reactions are readily detected by immunoblot analysis, these regions of PKC δ were not captured by our sequencing methods that achieved only ~40% coverage of the PKC δ sequence. Other PKC δ autophosphorylation sites may also have evaded detection and deserve further analysis. Third, PKC δ autophosphorylation at Thr 295 and Ser 304 was detected in kinase assays both without and with Src. These results indicate that PKC δ -Tyr 313 /Tyr 334 phosphorylation by Src is not required for PKC δ autophosphorylation at these sites. In contrast, PKC δ autophosphorylation at Thr 141 , Thr 218 , Ser 503 , and Ser 645 was detected exclusively in kinase assays with Src. The significance of this finding is uncertain. Our previous studies using immunoblot analysis showed that Src increases PKC δ autophosphorylation at Thr 505 and Thr 645 (Ref (14) and data not shown). Therefore, an effect of Src to increase PKC δ -Thr 505 autophosphorylation might lead to a general increase in PKC δ autophosphorylation throughout the protein. Alternatively, the failure to detect certain phospho-peptides in assays without Src may simply result from a bias inherent in the study; sequencing methods were disproportionately applied to peaks derived from assays with PKC δ plus Src (since PKC δ autophosphorylation was identified as a byproduct of studies designed to identify sites for Src-dependent PKC δ tyrosine phosphorylation).

As an initial approach to determine whether these novel phosphorylation sites are modified on PKC δ *in vivo*, mouse PKC δ was overexpressed in cardiomyocyte cultures metabolically

labeled with ^{32}P and then treated with H_2O_2 (to activate PKC δ and promote Src-dependent PKC δ tyrosine phosphorylation). PKC δ was then immunoprecipitated and subjected to phosphopeptide mapping analysis. A representative RP-HPLC chromatogram depicted in Fig 2B shows that PKC δ is phosphorylated at Thr 141 , Ser 504 , and Ser 662 (the hydrophobic motif) in H_2O_2 -treated cardiomyocytes. These studies provide important evidence that PKC δ -Thr 141 phosphorylation can be detected *in vivo* in cardiomyocytes. The identification of Ser 504 as a phosphoaccepter site was more surprising, since autophosphorylation at this site was not detected in the *in vitro* kinase assays. Ser 504 is an evolutionarily conserved phosphorylation site adjacent to the threonine phosphoaccepter site in the activation loops of PKC δ and aPKCs [ζ and λ/ι]; cPKCs or other nPKCs do not contain a phosphorylatable residue at this position. While it is interesting to speculate that this serine may play a redundant role with Thr 505 to structure the catalytic pocket of PKC δ for catalysis, our recent mutagenesis studies suggest otherwise, showing that a single T505A substitution is sufficient to prevent Src-dependent changes in PKC δ substrate specificity. Detailed studies that consider a possible role for Ser 504 to substitute for Thr 505 as a modification that regulates PKC δ 's substrate specificity are in progress.

Thr 141 contributes to the control of PKC δ downregulation in cells

Previous studies implicated autophosphorylations at residues N-terminal to the pseudosubstrate domain of PKC β II in the control of PKC β II activation. Since Thr 141 maps to a similar position in PKC δ , we used a heterologous overexpression strategy, with GFP-tagged WT-PKC δ and PKC δ constructs harboring non-phosphorylatable alanine or phospho-mimetic aspartate substitutions at position 141 to examine whether Thr 141 phosphorylation plays a similar role to influence PKC δ activation or downregulation. Fig 3A shows that WT-PKC δ , PKC δ -T141A, and PKC δ -T141D expression is similar in resting COS7 cells. WT-PKC δ and PKC δ -T141A are recovered primarily in the cytosolic fraction, but a smaller pool of WT-PKC δ and PKC δ -T141A constitutively localize to the particulate fraction. PKC δ -T141D also is primarily recovered in the cytosolic fraction. However, a 4.2 ± 0.5 -fold greater amount of PKC δ -T141D constitutively partitions to the particulate fraction, relative to WT-PKC δ ($n=5$, $p<0.05$). This increase in constitutive PKC δ -T141D partitioning to the particulate fraction is indicative of *in vivo* PKC δ activation. While a reciprocal decrease in PKC δ -T141D recovery in the soluble fraction is not obvious in Fig 3A, this is due to technical issues related to the detection of small differences in PKC δ immunoreactivity in soluble fractions that contain the bulk of the PKC δ immunoreactivity; the predicted differences in soluble PKC δ levels are resolved when protein loading for soluble fractions is decreased 5-fold (data not shown). Additional studies showed that lipid cofactors such as PMA or the cell-permeable DAG analog diC8 induce a similar complete translocation of all three enzymes from the soluble to the particulate fraction; no PKC δ , PKC δ -T141A, or PKC δ -T141D immunoreactivity remains in the soluble fraction of PMA- or diC8-treated COS7 cells.

The observation that a T141D substitution leads to the constitutive recruitment of greater amounts of PKC δ to the particulate fraction suggests that a phospho-mimetic substitution at Thr 141 favors PKC δ activation. This conclusion gains support from studies that examine the kinetics of PMA-dependent PKC δ downregulation. While WT-PKC δ , PKC δ -T141A, and PKC δ -T141D were consistently recovered at similar levels under resting culture conditions (arguing that the Thr 141 substitution does not lead to a gross change in the stability of the enzyme), Fig 3B exposes differences in the kinetics of PKC δ downregulation. PMA treatment leads to a time-dependent decrease in WT-PKC δ abundance; WT-PKC δ abundance falls over the first 4 hr of PMA treatment and remains at this reduced level for the next 20 hr. While these results are at odds with early studies showing that chronic PMA treatment leads to the complete downregulation of PKC δ , more recent studies with newer lots of anti-PKC δ antibodies (that are considerably more sensitive than the reagents previously available) indicate that substantial

amounts of residual PKC δ immunoreactivity can be detected in various cells types treated with PMA for 24 hr. PMA treatment also leads to a time-dependent decline in PKC δ -T141A abundance. However, PMA-dependent PKC δ -T141A downregulation is delayed compared to PMA-dependent downregulation of WT-PKC δ . Of note, residual levels of WT-PKC δ and PKC δ -T141A are similar in COS7 cells treated with PMA for 24 hr. In contrast, PMA treatment leads to a considerably more rapid and complete downregulation of PKC δ -T141D. PKC δ -T141D abundance falls dramatically following a 2 hr incubation with PMA, and only trace amounts of PKC δ -T141D immunoreactivity are detected in COS7 cells treated with PMA for 4 hr; PKC δ -T141D immunoreactivity is not detected in COS7 cells treated with PMA for 24 hr. These results are consistent with the notion that a T141A substitution stabilizes the closed conformation of the enzyme and prevents activation/downregulation and that an autophosphorylation (or a negative charge) in the vicinity of the PKC δ pseudosubstrate domain lowers pseudosubstrate domain binding affinity for the catalytic pocket and favors activation.

Thr²⁹⁵ autophosphorylation regulates *in vitro* PKC δ catalytic activity

GFP-tagged WT-PKC δ and PKC δ -T295A, and PKC δ -T141A were overexpressed in COS7 cells and immunoprecipitated for use in kinase assays to determine whether Thr²⁹⁵ and Thr¹⁴¹ contribute to the control of PKC δ catalytic function. Kinase assays were performed with cTnI as substrate - without and with Src - to determine whether Thr²⁹⁵ and Thr¹⁴¹ influence PKC δ -Thr⁵⁰⁵ phosphorylation, PKC δ tyrosine phosphorylation by Src, and/or PKC δ phosphorylation of cTnI. Assays also were performed over a range of ATP concentrations to examine whether these phosphorylation sites influence the ATP requirements for PKC δ autophosphorylation or PKC δ phosphorylation of cTnI. Fig 4A shows that WT-PKC δ , PKC δ -T295A, and PKC δ -T141A are recovered as constitutively Thr⁵⁰⁵-phosphorylated enzymes, indicating that T295A and T141A substitutions do not disrupt *in vivo* PKC δ -Thr⁵⁰⁵ phosphorylation. While we have not yet generated phospho-site specific antibodies (PSSAs) to track PKC δ phosphorylation at Thr²⁹⁵ or Thr¹⁴¹, we noted that Thr²⁹⁵ is flanked by a +2 position Arg (i.e., T²⁹⁵QR); the Cell Signaling Technologies anti-pTXR PSSA specifically recognizes this phosphorylation motif. Fig 4 shows that WT-PKC δ and PKC δ -T141A are recovered from COS7 cells without any appreciable anti-pTXR immunoreactivity co-migrating with PKC δ . The anti-pTXR PSSA detects *in vitro* WT-PKC δ and PKC δ -T141A autophosphorylation; this autophosphorylation reaction is detected at 1 μ M ATP and is maximal at 6 μ M ATP. The anti-pTXR PSSA does not detect autophosphorylation of the PKC δ -T295A mutant. The observation that WT-PKC δ and PKC δ -T141A undergo similar *in vitro* autophosphorylation reactions at a site recognized by the anti-pTXR PSSA, whereas the PKC δ -T295A mutant does not, provides strong evidence that the anti-pTXR PSSA specifically recognizes *in vitro* PKC δ autophosphorylation at PKC δ -Thr²⁹⁵. While an effect of the T295A substitution to influence anti-TXR immunoreactivity indirectly by controlling PKC δ autophosphorylation reaction a different TXR motif in PKC δ can not formally be excluded, other TXR motifs at T³⁸ER and T⁵⁸⁸KR in human PKC δ have not been identified as autophosphorylation sites; this alternative interpretation of the results is considered substantially less likely. Since the anti-TXR PSSA appears to track PKC δ -Thr²⁹⁵ autophosphorylation, it was used to examine whether PKC δ -Thr²⁹⁵ autophosphorylation is dynamically regulated *in vivo* in COS7 cells. Fig 4B shows that WT-PKC δ and PKC δ -T295A constructs are recovered in similar amounts, without any basal anti-TXR immunoreactivity, from vehicle-treated COS7 cells. The anti-TXR antibody detects a PMA-dependent increase in WT-PKC δ phosphorylation, whereas no PMA-dependent increase in PKC δ -T295A phosphorylation is detected. These results suggest that Thr²⁹⁵ phosphorylation also accompanies PKC δ activation *in vivo* in a cellular context.

In vitro kinase assays were performed in the presence of Src, as a strategy to further interrogate the mechanisms that regulate PKC δ activity. Fig 4A shows that WT-PKC δ and PKC δ -T141A

are phosphorylated by Src at Tyr³¹¹ in a similar manner; Src (and PKC δ -Tyr phosphorylation) does not lead to any obvious change in PKC δ autophosphorylation at Thr²⁹⁵. However, the PKC δ -T295A construct (harboring a T→A substitution in the vicinity of Tyr³¹¹ in the hinge region) shows a relative defect in Src-dependent Tyr³¹¹ phosphorylation. The functional consequences of altered Thr²⁹⁵ autophosphorylation and Src-dependent Tyr³¹¹ phosphorylation were examined in kinase assays with cTnI as a substrate. cTnI phosphorylation was tracked by immunoblot analysis with an anti-cTnI-pSer²³/Ser²⁴ PSSA that specifically recognizes the Ser²³/Ser²⁴-phosphorylated form of cTnI and the Cell Signaling anti-pTXR PSSA that selectively recognizes cTnI phosphorylation at KRPT¹⁴⁴LR (as validated in (4)). Allosterically-activated forms of WT-PKC δ , PKC δ -T295A, and PKC δ -T141A (in assays with PS/PMA) phosphorylate cTnI at Ser²³/Ser²⁴ in a similar manner. In each case, cTnI-Ser²³/Ser²⁴ phosphorylation is detected at 1 μ M ATP and maximal at 6 μ M ATP. WT-PKC δ and PKC δ -T141A become cTnI-Thr¹⁴⁴ kinases in assays with Src. Control experiments indicate that cTnI-Thr¹⁴⁴ phosphorylation can not be attributed to a contaminant with serine/threonine kinase activity in the Src preparation, since *in vitro* kinase assays with Src and cTnI (without PKC δ) do not lead to any detectable ³²P-incorporation into cTnI by PhosphorImager or evidence of cTnI phosphorylation by immunoblot analysis with the anti-cTnI-Thr¹⁴⁴ PSSA. Of note, a T295A substitution decreases the cTnI-Thr¹⁴⁴ kinase activity of the PKC δ enzyme. These results are consistent with previous studies showing that the effect of Src to convert PKC δ into a cTnI-Thr¹⁴⁴ kinase can be abrogated by a Y311F substitution; the PKC δ -T295A mutant displays a defect in Src-dependent Tyr³¹¹ phosphorylation and decreased cTnI-Thr¹⁴⁴ kinase activity. These results identify a novel PKC δ autophosphorylation reaction at Thr²⁹⁵ (a highly conserved residue in the hinge region) that is critical for Src-dependent PKC δ -Tyr³¹¹ phosphorylation and consequently Src-dependent changes in PKC δ substrate specificity.

We previously reported that PKC δ acts as a cTnI-Ser²³/Ser²⁴ kinase in assays with PS/PMA and as a dual Ser²³/Ser²⁴ and Thr¹⁴⁴ kinase in assays with PS/PMA plus Src, and that a single T505A substitution prevents the Src-dependent acquisition of cTnI-Thr¹⁴⁴ kinase activity. These studies implicated Thr⁵⁰⁵ autophosphorylation as a modification that critically regulates PKC δ phosphorylation of a heterologous substrate. The next set of experiments examined whether Thr⁵⁰⁵ phosphorylation regulates PKC δ autophosphorylation at Thr²⁹⁵. Fig 5 shows that WT-PKC δ is recovered as a constitutively Thr⁵⁰⁵-phosphorylated enzyme that undergoes an autophosphorylation reaction at Thr²⁹⁵ in assays performed with PS/PMA. WT-PKC δ phosphorylates cTnI at Ser²³/Ser²⁴ in assays with PS/PMA, and at Ser²³/Ser²⁴ and Thr¹⁴⁴ in assays with PS/PMA and Src. The PKC δ -T505A construct also undergoes an autophosphorylation reaction in assays with PS/PMA (data not shown); PKC δ -T505A phosphorylates cTnI at Ser²³/Ser²⁴ in assays with PS/PMA (similar to WT-PKC δ). However, PKC δ -T505A autophosphorylation at Thr²⁹⁵ and Tyr³¹¹ phosphorylation by Src are defective, relative to WT-PKC δ ; Src does not convert the PKC δ -T505A mutant into a cTnI-Thr¹⁴⁴ kinase. Collectively, these results suggest a novel model for PKC δ activation. Our results indicate that PKC δ -Thr⁵⁰⁵ autophosphorylation has no effect on some aspects of catalytic activity (for example cTnI-Ser²³/Ser²⁴), but it is required for optimal PKC δ autophosphorylation at Thr²⁹⁵ and Src-dependent PKC δ -Tyr³¹¹ phosphorylation, a modification that converts PKC δ into a cTnI-Thr¹⁴⁴ kinase.

A T141D substitution favors *in vitro* PKC δ activation

In the final set of experiments, GFP-tagged WT-PKC δ and PKC δ -T141D were overexpressed in COS7 cells and immunoprecipitated for use in kinase assays to determine whether a negative charge at Thr¹⁴¹ favors PKC δ activation. Fig 6A (*top*) shows that WT-PKC δ and PKC δ -T141D constructs are expressed at similar levels in COS7 cells and that an immunoprecipitation with an anti-GFP antibody (that recognizes the tag on the heterologously overexpressed PKC δ enzyme) completely clears these enzymes from COS7 cell lysates. Similar amounts of WT-

PKC δ and PKC δ -T141D proteins are recovered in anti-GFP pulldowns (Fig 6A, *bottom*). WT-PKC δ and PKC δ -T141D enzymes were then used in kinase assays without and with PS/PMA (in the absence or presence of Src). Fig 6B shows that WT-PKC δ and PKC δ -T141D are recovered with similar levels of constitutive phosphorylation at Thr⁵⁰⁵, but not Thr²⁹⁵ (i.e., a T141D substitution that increases PKC δ partitioning to the particulate fraction does not lead to an increase in basal PKC δ -Thr²⁹⁵ autophosphorylation in COS7 cells). However, the PKC δ -T141D mutant displays a substantial amount of lipid-independent kinase activity that is not detected in assays with the WT-PKC δ enzyme. PKC δ -T141D executes a robust lipid-independent autophosphorylation reaction, detected as both an increase in Thr²⁹⁵ autophosphorylation by immunoblot analysis and an increase in ³²P-incorporation into the mutant enzyme by PhosphorImager analysis. The PKC δ -T141D mutant also is a lipid-independent cTnI kinase. PKC δ -T141D activity is only slightly further increased when assays are performed in the presence of PS/PMA. The Src-dependent increase in Y³¹¹ phosphorylation is similar for WT-PKC δ and PKC δ -T141D enzymes. These results indicate that a negative charge at Thr¹⁴¹ (in the vicinity of the pseudosubstrate domain) disrupts autoinhibitory interactions that restrict basal PKC δ activity.

DISCUSSION

This study identifies seven novel PKC δ autophosphorylation sites in very distinct regions of PKC δ at Thr¹⁴¹ adjacent to the pseudosubstrate motif, Thr²¹⁸ in the C1A–C1B interdomain region, Thr²⁹⁵, Ser³⁰², and Ser³⁰⁴ in the hinge region, and two serine residues (S⁵⁰¹RAS⁵⁰⁴T) N-terminal to the activation loop Thr⁵⁰⁵ phosphorylation site. While these novel autophosphorylation sites are evolutionarily conserved in PKC δ , they do not reside in specific phosphorylation motifs common to all PKC isoforms. Rather, these novel autophosphorylation sites map to functionally important regions of the protein that appear to be autophosphorylation ‘hot spots’ in other PKC isoforms. For example, PKC δ and PKC ϵ contain sites for autophosphorylation in the C1A–C1B interdomain region, although the PKC δ C1A–C1B interdomain autophosphorylation site (NSRDT²¹⁸IF) bears no resemblance to the C1A–C1B interdomain autophosphorylation site recently identified in PKC ϵ (PDQVGS²³⁴QR). These results suggest that PKCs may have evolved to contain sites for autophosphorylation at key positions (rather than conserved phosphorylation motifs) in the enzyme. In fact, one could speculate that differences in the autophosphorylation motifs identified in individual nPKC isoforms may have evolved to accommodate the distinct catalytic requirements of these enzymes.

In the course of these studies, two other laboratories identified seven novel sites for PKC δ autophosphorylation at Thr⁵⁰, Thr¹⁴¹, Ser²⁹⁹, Ser³⁰², Ser³⁰⁴, Thr⁴⁵¹, and Ser⁵⁰⁶ (human numbering) (10;15). Studies from the Parker laboratory suggest that Ser²⁹⁹ autophosphorylation may provide a convenient marker for PKC δ activation, that could be used (in lieu of more cumbersome methods that assay for PKC δ translocation to membranes) to screen for PKC δ activation in tumor models. The notion that PKC δ autophosphorylation might play a functionally important role to regulate catalysis was not considered. Of the four autophosphorylation sites at Thr¹⁴¹, Ser³⁰², Ser³⁰⁴ and Ser⁵⁰⁶ that were detected in both the previous publication and our study, Thr¹⁴¹ is particularly interesting. Thr¹⁴¹ is strategically positioned N-terminal to the pseudosubstrate domain, a region that maintains PKC δ in an inactive conformation through an intramolecular interaction with the catalytic pocket. Our mutagenesis studies show that PKC δ is phosphorylated at Thr¹⁴¹ *in vivo* in COS7 cells and that Thr¹⁴¹ autophosphorylation influences the kinetics of PKC δ activation/downregulation in cells. The PKC δ -T141D mutant is constitutively recovered (in greater amounts than WT-PKC δ) in the particulate fraction; a T141A substitution slows, and a T141D substitution accelerates, the tempo of PKC δ downregulation. *In vitro* kinase assays provide further evidence that a phosphorylation reaction at Thr¹⁴¹ relieves autoinhibitory constraints that limit PKC δ

activity, showing that the PKC δ -T141D enzyme functions as a lipid-independent serine/threonine kinase. The conclusion that an autophosphorylation at Thr¹⁴¹ regulates PKC δ activity resonates with previous modeling studies of PKC β II, which proposed that negative charges due to autophosphorylation reactions at Ser¹⁶/Thr¹⁷ and an Arg residue at position 19 (a position that corresponds to Thr¹⁴¹ in PKC δ) participate in intramolecular binding interactions that influence enzyme activity.

Three autophosphorylation sites described in previous studies (at Thr⁵⁰, Ser²⁹⁹, and Thr⁴⁵¹) were not detected in our experiments (although one site was detected in the non-phosphorylated KPT⁵⁰MY⁵²PEWK peptide fragment, which contains putative phosphorylation sites at both Thr⁵⁰ and Tyr⁵²). Rather, this study identifies novel PKC δ autophosphorylation reactions at Thr²¹⁸ and Thr²⁹⁵, sites not detected in previous studies. Thr²¹⁸ resides in the C1A–C1B interdomain region, within a phosphorylation motif that is conserved in the C1A–C1B interdomain region of PKC θ . Studies of PKC θ implicate this C1A–C1B interdomain autophosphorylation reaction in mechanisms required for proper PKC θ targeting to lipid rafts and antigen receptor signaling responses in Jurkat and T cells (13). While the phosphorylation motif detected in PKC δ and PKC θ is not conserved in other PKC isoforms, regulatory autophosphorylation reactions have been identified in the C1A–C1B interdomain regions of PKC ϵ and PKD1 (the founding member of a different family of PMA/DAG-sensitive kinases (16)). These results suggest that the C1A–C1B interdomain region may be an autophosphorylation ‘hot spot’ that is exposed on the surface and regulates the localization/actions of these enzymes in cells.

Finally, this study characterizes the variable V3 hinge region of PKC δ as a target for functionally important tyrosine and serine/threonine phosphorylations. We recently reported that Tyr³¹¹ and Tyr³³² are the major sites for *in vitro* Src-dependent PKC δ phosphorylation. We also showed that PKC δ is dually phosphorylated at Tyr³¹¹ and Tyr³³² in cardiomyocytes subjected to oxidative stress. Recent studies implicate Tyr³¹¹ phosphorylation as a modification that modulates PKC δ catalytic activity toward Thr¹⁴⁴ on cTnI. Phosphorylation at Tyr³³² does not contribute to the control of kinase activity, but rather generates a docking site for PKC δ binding partners such as the adapter protein Shc (17). Studies reported herein (along with recent results from the Parker laboratory (10)) extend the analysis by showing that the human PKC δ hinge region contains a cluster of autophosphorylation sites at Thr²⁹⁵, Ser²⁹⁹, Ser³⁰² and Ser³⁰⁴. These sites, and their flanking sequences, are highly evolutionarily conserved in PKC δ ; they are not conserved in other mammalian PKC isoforms (although the hinge regions of PKC β II and PKC ϵ contain their own distinctive autophosphorylation motifs). The Parker laboratory detected PKC δ -Ser²⁹⁹ phosphorylation in a dually phosphorylated ₂₉₈ApS²⁹⁹RRpS³⁰²DS³⁰⁴ASSEPVGIIY³¹³QGFEK³¹⁸ peptide liberated by a 4 hr trypsin digest. Ser²⁹⁹ phosphorylation was not detected in our experiments that used a lengthier trypsin digestion (16 hr), which cleaved the 298–318 peptide fragment into a singly phosphorylated ₃₀₁RS³⁰²DS³⁰⁴ASSEPVGIIY³¹³QGFEK³¹⁸ peptide (with phosphorylation at either Ser³⁰², Ser³⁰⁴, or Tyr³¹³) and a very small ₂₉₈ASR³⁰⁰ fragment that was not captured in the experiments. The Parker laboratory identified a dynamic increase in PKC δ -Ser²⁹⁹ phosphorylation in the membrane compartment of PMA-treated COS7 or HeLa cells; a role for PKC δ -Ser²⁹⁹ phosphorylation to regulate PKC δ 's cellular or *in vitro* catalytic function was not considered. Studies reported herein focused on PKC δ autophosphorylation at Thr²⁹⁵, a different autophosphorylation reaction that was not detected in the previous study. *In vitro* kinase assays exposed a novel role for PKC δ -Thr²⁹⁵ autophosphorylation as a lipid-dependent modification that links PKC δ -Thr⁵⁰⁵ autophosphorylation to PKC δ regulation by Src. PKC δ -Thr²⁹⁵ autophosphorylation is reduced in the PKC δ -T505A mutant and a T295A substitution leads to a defect in Src-dependent PKC δ -Tyr³¹¹ phosphorylation. These results provide a molecular explanation for the previous observation that Src phosphorylates PKC δ at Tyr³¹¹ only when assays are performed in the presence of lipid cofactors. We had previously

speculated that lipid cofactors induce a conformational change that renders PKC δ a better substrate for Src. However, studies reported herein expose an additional effect of PMA or DAG to trigger a series of ordered autophosphorylation reactions at Thr⁵⁰⁵ and Thr²⁹⁵ that are required for Src-dependent PKC δ -Tyr³¹¹ phosphorylation (schematized in Fig 7). While a mechanism whereby an autophosphorylation reaction at Thr²⁹⁵ would prime PKC δ for subsequent Tyr³¹¹ phosphorylation by Src is uncertain, these studies suggest that compounds targeted to the PKC δ -Thr²⁹⁵ autophosphorylation site might selectively interdict the catalytic function of Tyr³¹¹-phosphorylated PKC δ , without acting as general inhibitors of the enzyme. Since there is evidence that the hinge region of PKC β II is exposed (and becomes proteolytically labile) in the active conformation of PKC β II (8), the hinge region of PKC δ also is likely to provide an accessible surface for drug interactions. A compound that inhibits PKC δ -Thr²⁹⁵ phosphorylation would constitute a novel class of PKC δ inhibitors that would selectively prevent the cellular actions of tyrosine phosphorylated PKC δ (in the soluble fraction of cells exposed to oxidative stress), while preserving PKC δ actions/phosphorylations that result from GPCR activation and the generation of DAG in membranes.

PKC isoforms play key roles in mechanisms that regulate cell proliferation, survival, and migration. PKCs are dysregulated in many clinically important disorders and genetic studies in PKC knockout models in mice provide compelling evidence that PKC isoforms contribute to the pathogenesis of various immune disorders, atherosclerotic and diabetic cardiovascular diseases, and malignancies. Current methods to screen for PKC activation are quite limited. Most studies have relied on measurements of PKC protein expression, which typically correlates at best only weakly with PKC activation. While measurements of PKC translocation to membranes are more informative, translocation studies are too cumbersome to use as screens for PKC activation (and translocation provides an imperfect measure of enzyme activation under certain circumstance, since some PKCs are activated in the cytosol via non-traditional lipid-independent mechanisms during oxidative stress (18)). Studies to determine whether Thr²⁹⁵ in PKC δ 's hinge region is phosphorylated exclusively via an obligate intramolecular autocatalytic reaction will be critical to determine whether this modification can be exploited as a biomarker to screen for PKC δ activation in biologically relevant tissue samples.

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ABBREVIATIONS

cTnI	cardiac troponin I
DAG	diacylglycerol
diC8	1,2-dioctanoylglycerol
EGFP	enhanced green fluorescent protein
ESI-LC/MS/MS	

electrospray ionization-liquid chromatography/tandem mass spectrometry

PDK-1

phosphoinositide dependent kinase 1

PKC

Protein kinase C

aPKC

atypical protein kinase C

cPKC

conventional protein kinase C

nPKC

novel protein kinase C

PKD1

protein kinase D1

PMA

phorbol 12-myristate 13-acetate

PS

phosphatidylserine

PSSA

phospho-site specific antibody

RP-HPLC

reverse phase-high pressure liquid chromatography

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Fig. 1. Schematic of autophosphorylation sites adjacent to the pseudosubstrate domain, in the C1A–C1B interdomain region and in the hinge region of PKCδ

Domain structure of PKCδ with conserved regions (C2-like and C1A–C1B) regions in the regulatory domain, conserved (C3 and C4) regions in the catalytic domain. The PKCδ autophosphorylation sites identified in this study are located N-terminal to the pseudosubstrate (PS) domain sequence (which is underlined in the figure), in the C1A–C1B interdomain and in the hinge region. PKCδ tyrosine phosphorylation sites at position Tyr¹⁵⁵ (C-terminal to the pseudosubstrate domain sequence) and at tyrosine residues in the hinge region also are indicated. Pseudosubstrate and C1A–C1B interdomain regions of PKCδ are evolutionarily conserved; species differences in the hinge region sequences of human (h), rat (r), and mouse (m) PKCδ are provided.

Fig. 2. PKCδ autophosphorylation at Thr¹⁴¹, Thr²¹⁸, Thr²⁹⁵, Ser³⁰², Ser³⁰⁴, and Thr⁵⁰³
Panel A: PKCδ was incubated in a kinase buffer containing [³²γP]-ATP without and with active Src and PKCδ phosphorylation was tracked by and ESI-LC/MS/MS analysis according to Experimental Procedures. *Panel B:* Cardiomyocytes cultures were metabolically labeled

with ^{32}P and then treated for 10 min with vehicle or 5 mM H_2O_2 . Heterologously overexpressed mouse PKC δ was immunoprecipitated from the cultures and then subjected to phosphopeptide mapping analysis as described in Experimental Procedures.

Fig. 3. An autophosphorylation reaction at Thr¹⁴¹ regulates the kinetics of PKC δ activation and downregulation in COS7 cells

Panel A: COS7 cells that heterologously overexpress GFP-tagged WT-PKC δ , PKC δ -T141A, and PKC δ -T141D were treated for 20 min with vehicle, 200 nM PMA or 200 nM diC8. A small aliquot of cell lysate was retained for Western blotting studies to validate that WT-PKC δ , PKC δ -T141A, and PKC δ -T141D expression levels are similar in resting COS7 cells (*left*). The remainder of the cell lysate was then partitioned into soluble and particulate fractions according to Experimental Procedures and then immunoblotting studies were performed to track PKC δ fractionation between soluble and particulate fractions (*right*). Since PKC δ was expressed as a fusion protein with EGFP, PKC δ was detected with an anti-GFP antibody; no band is identified in uninfected cultures (data not shown). Results are typical of data obtained in 5 separate experiments. *Panel B:* COS7 cells that heterologously overexpress WT-PKC δ ,

PKC δ -T141A, and PKC δ -T141D were treated with vehicle for 24 hr or with 200 nM PMA for the indicated intervals; short incubations with PMA were initiated with a delay, so that all samples were harvested simultaneously (and the total treatment interval was similar for all samples). The kinetics of PKC δ protein downregulation was tracked by immunoblot analysis; immunoblotting for ERK is included to control for any minor differences in sample loading. The immunoblotting data and quantification are from a single experiment, with similar results in two separate experiments.

Fig 4. PKC δ autophosphorylation at Thr²⁹⁵ is required for Src-dependent PKC δ -Tyr³¹¹ phosphorylation and PKC δ -dependent cTnI phosphorylation at Thr¹⁴⁴

Panel A: COS7 cells were transfected with plasmids that drive expression of WT- and T295A- or T141A-substituted forms of PKC δ fused to GFP. PKC δ was immunoprecipitated with anti-GFP, subjected to immunoblotting with anti-GFP to validate equal protein recovery (*left*). Equal amounts of enzyme were then subjected to immuno-complex kinase assays without and with lipid cofactors, Src, or different ATP concentrations as indicated (*right*). Immunoblot analysis was used to track PKC δ phosphorylation at Thr⁵⁰⁵, Thr²⁹⁵ (detected with the anti-pTXR PSSA), and Tyr³¹¹ as well as cTnI phosphorylation at Ser²³/Ser²⁴ and Thr¹⁴⁴ (detected with the anti-pTXR PSSA). *Panel B:* COS7 cells were transfected with plasmids that drive expression of WT-PKC δ or PKC δ -T295A fused to GFP. PKC δ was immunoprecipitated with anti-GFP and subjected to immunoblotting with anti-GFP to validate equal protein recovery (*bottom*) and anti-TXR immunoreactivity (to track Thr²⁹⁵ phosphorylation).

Fig 5. The PKC δ -T505A mutant displays a defect in PKC δ autophosphorylation at Thr²⁹⁵ and PKC δ -dependent cTnI phosphorylation at Thr¹⁴⁴

Panel A: COS7 cells were transfected with plasmids that drive expression of WT- and T505A-substituted forms of PKC δ fused to GFP. PKC δ was immunoprecipitated with anti-GFP, subjected to immuno-complex kinase assays without and with lipid cofactors or Src (*top*) and immunoblotting with anti-GFP to validate equal protein recovery (*bottom*). PKC δ and cTnI phosphorylation were detected as described in the legend to Fig 4. Results were replicated in two additional separate experiments.

Fig 6. A T141D substitution activates PKCδ

COS7 cells were transfected with plasmids that drive expression of WT-PKCδ or PKCδ-T141D fused to GFP; PKCδ was then immunoprecipitated with anti-GFP. *Panel A:* Immunoblotting of cell lysates to show that WT-PKCδ and PKCδ-T141D expression levels are similar and that the anti-GFP immunoprecipitation protocol clears both enzymes from cell lysates (*top*). Immunoblotting was performed on increasing amounts of protein recovered in anti-GFP pull-downs, to enhance our ability to detect even minor differences in WT-PKCδ versus PKCδ-T141D protein recovery. The Western blot in Fig 6A (*bottom*) shows that equivalent amounts of WT-PKCδ and PKCδ-T141D were recovered in the anti-GFP pull-downs. *Panel B:* Equal amounts of WT-PKCδ and PKCδ-T141D were subjected to immuno-complex kinase assays without and with lipid cofactors or Src. Immunoblot analysis was used to track PKCδ phosphorylation at Thr⁵⁰⁵, Thr²⁹⁵ (detected with the anti-pTXR PSSA), and Tyr³¹¹ (*top*). ³²P-incorporation into PKCδ and cTnI also was tracked by PhosphorImager analysis (*bottom*). Results are from a single experiment and are representative of three independent experiments with similar results.

Fig 7. Schematic of the controls of PKC δ phosphorylation and activity toward cTnI
See text.

Table 1

Summary of the phosphopeptides derived from PKC δ detected by ESI-LC/MS/MS analysis from *in vitro* kinase assays with PKC δ alone or PKCS plus Src

SITE	PHOSPHOPEPTIDE SEQUENCE	DETECTED IN ASSAYS WITH PMA	DETECTED IN ASSAYS WITH PMA+SRC
pT ¹⁴¹	₁₃₉ FPpT ¹⁴¹ MNR ₁₄₄		+
pT ²¹⁸	₂₁₇ DpT ²¹⁸ IFQK ₂₂₂		+
pT ²⁹⁵	₂₈₆ LLAEALNQVpT ²⁹⁵ QR ₂₉₇	+	+
pS ³⁰²	₃₀₁ RpS ³⁰² DSASSEPVGIYQGFEK ₃₁₈ ₃₀₂ pS ³⁰² DSASSEPVGIYQGFEK ₃₁₈	+	+
pS ³⁰⁴	₃₀₁ RSDpS ³⁰⁴ ASSEPVGIYQGFEK ₃₁₈ ₃₀₂ SDpS ³⁰⁴ ASSEPVGIYQGFEK ₃₁₈	+	+
pY ³¹³	₃₀₁ RSDSASSEPVGIpY ³¹³ QGFEK ₃₁₈ ₃₀₂ SDSASSEPVGIpY ³¹³ QGFEK ₃₁₈		+
pY ³³⁴	₃₂₀ TGVAGEDMQDNSGTpY ³³⁴ GK ₃₃₆		+
pS ⁵⁰³	₄₉₇ ENIFGEpS ⁵⁰³ R ₅₀₄		+
pS ⁶⁴⁵	₆₄₁ KARLpS ⁶⁴⁵ YSDKNLIDSMOXDQ ₆₅₇		+

NOTE: Y³¹³ in human PKC δ corresponds to Y³¹¹ in the rodent PKC δ sequence.