Activation loop phosphorylation plays critical regulatory roles for many kinases. Unlike other protein kinase Cs (PKC), PKC-δ does not require phosphorylation of its activation loop (Thr-507) for \textit{in vitro} activity. We investigated the structural basis for this unusual capacity and its relevance to PKC-δ function in intact cells. Mutational analysis demonstrated that activity without Thr-507 phosphorylation depends on 20 residues N-terminal to the kinase domain and a pair of phenylalanines (Phe-500/Phe-527) unique to PKC-δ near the activation loop. Molecular modeling demonstrated that these elements stabilize the activation loop by forming a hydrophobic chain of interactions from the C-lobe to activation loop to N-terminal (helical) extension. In cells PKC-δ mediates both apoptosis and transcription regulation. We found that the T507A mutant of the PKC-δ kinase domain resembled the corresponding wild type in mediating apoptosis in transfected HEK293T cells. But the T507A mutant was completely defective in AP-1 and NF-κB reporter assays. A novel assay in which the kinase domain of PKC-δ and its substrate (a fusion protein of PKC substrate peptide with green fluorescent protein) were co-targeted to lipid rafts revealed a major substrate-selective defect of the T507A mutant in phosphorylating the substrate in cells. \textit{In vitro} analysis showed strong product inhibition on the T507A mutant with particular substrates whose characteristics suggest it contributes to the substrate selective defect of the PKC-δ T507A mutant in cells. Thus, activation loop phosphorylation of PKC-δ may regulate its function in cells in a novel way.

Protein kinase C (PKC) is a family of 9 genes that can be further divided into classical, novel, and atypical PKCs, depending on their structural characteristics and their requirement for activation (1, 2). Each of them is autoinhibited by an intramolecular interaction of the kinase domain with an N-terminal regulatory domain, whose organization differs between subfamilies. Classic PKCs have C1 and C2 domains that bind diacylglycerol and Ca$^{2+}$, respectively, for their activation. Novel PKCs have a C1 domain that binds diacylglycerol, but their C2-like domain does not bind Ca$^{2+}$. Atypical PKCs do not have the ability to bind either Ca$^{2+}$ or diacylglycerol, but are activated by other lipids or small G-proteins. Binding of the regulatory region with appropriate cofactors causes a conformational change that releases the auto-inhibition and results in activation.

Besides the co-factor-induced conformational change, PKC activity is also regulated by phosphorylation on its kinase domain, most importantly on its activation loop (3, 4). The activation loop is a stretch of 20–30 amino acids located in the catalytic cleft of the kinase domain of all eukaryotic protein kinases that form part of the substrate peptide binding surface. The activation loop is relatively flexible, and undergoes varied forms of conformation regulation between the active and inactive states (5–7). One of the most common modes of kinase regulation is by phosphorylation of residues in the activation loop (8, 9). When phosphorylated, the negatively charged phosphate forms critical interactions with charged residues on the kinase domain, stabilizing an active conformation. In some cases, there are no phosphorylation sites on the activation loop, but instead a negatively charged residue serves as a functional replacement for the phosphate group. There are also kinases that do not need negative charge on the activation loop to be active. Generally, PKCs depend on activation loop phosphorylation for their kinase activity. It is believed that most PKCs are well phosphorylated at its kinase domain shortly after synthesis (2, 4). So most cellular PKC is “competent,” but its activity is autoinhibited until it is released by a conformational change caused by co-factor binding.

But PKC-δ is a special case; the dependence of its activity on activation loop phosphorylation is still a point of controversy. Some studies have found that the activation loop-dephosphorylated mutant of PKC-δ was still fully active in the \textit{in vitro} kinase assay (10–12). Others found that PKC-δ only had minimal activity when not phosphorylated, and its activity increased about 10-fold when phosphorylated on its activation loop (13). Although various possible explanations have been proposed, the cause of the discrepancy is still unclear. In contrast to PKC-δ, the catalytic activity of the closest isofrom, PKC-ε, is strictly dependent on activation loop phosphorylation (12). Because the two kinases have 87% sequence similarity in the kinase domain, they constitute an informative pair for investigating the structural basis of the unique functional properties of PKC-δ.

As noted above, activation loop phosphorylation is generally constitutive in novel and classical PKCs in cells. In contrast, although PKC-δ is constitutively phosphorylated on its activation loop in some cell types, in a variety of contexts it undergoes regulated activation loop phosphorylation or dephosphorylation in cells (13–16), which therefore resembles the regulatable phosphorylation that is so critical for many other kinases, including AGC kinases such as PKC-ζ, PRK1, and AKT. It is appealing that this phosphorylation has an important regulatory role in cells, but that possibility has been clouded by the controversy regarding the functional effect of phosphorylation at this site.

We therefore investigated in greater detail the structural basis of activity regulation by phosphorylation of Thr-507 and the functional...
consequences thereof. Using a combination of mutational analysis, structural modeling, and molecular dynamics we have identified the molecular basis for stabilization of the activation loop of PKC-δ in the absence of phosphorylation. Moreover, our assessment of its functional capacity demonstrates both an altered range of substrates in cells and increased susceptibility to product inhibition in vitro. Of particular interest, we find that phosphorylation of Thr-507 is not required for apoptosis, one of the most notable functions of PKC-δ in cells.

MATERIALS AND METHODS

DNA Constructs—PKC-δ cDNA constructs have been described previously (12). PKC-δ kinase domain and substrate (MARCKS and CREB) constructs were created using the Gateway cloning system (Invitrogen). The kinase domains (with or without the 20-residue N-terminal extension) are from human protein: PKC-δ (aa 329–676 or 349–676). Targeted substrate constructs were made by cloning the annealed oligonucleotides coding for the following peptide sequence into the pDEST516 Gateway destination vector with Lck targeting motif and HA tag at the N-terminal and green fluorescent protein tag at the C terminus: human MARCKS aa 154–168 (KKKRFSFKSKFLSG) and CREB aa 126–140 (EILSRRPSYRKILND) (supplemental Fig. 6A).

Antibodies and Peptides—Anti-HA tag mAb and HA peptide were purchased from BabCO; anti-PKC-δ pT507, phospho-MARCKS and CREB antibodies were from Cell Signaling Technologies. MLK3 (QQVDRERPHVRARRGpTFKRS) and PARIS1 (EYLLRapsRRRAV) phosphopeptides and the corresponding non-phosphopeptide were synthesized by Peptron Inc.; PKC-α pseudo-substrate peptide was purchased from Biomet. The panel of 96 proteomic peptides was synthesized by AxCell Biosciences as previously described (17).

Transfection, Apoptosis Assay, Immunoprecipitation, and Western Blot—HEK293T cell transfection was done with calcium phosphate using standard procedures. For apoptosis assay, 20 h after transfection the cells were fixed with 4% paraformaldehyde, permeabilized, stained with HA mAb and then 20 μg of HA mAb and eluted from streptavidin-coated plates as described previously (17). Kₘ for ATP was assessed using PKC-α pseudo-substrate peptide as substrate and ATP concentrations ranging from 0.25 to 400 μM.

In-cell Phosphorylation Assay—Lck motif-targeted PKC-δ kinase domain constructs and substrate (MARCKS and CREB) constructs were co-transfected into HEK293T cells. 24 h after transfection, the cells were harvested and lysed. The cell lysate was blotted with HA-tagged mAb, to determine expression of both kinase domain and substrate constructs, and pMARCKS/pCREB antibody, to determine phosphorylation of the substrates. For all assays, all results shown are representatives of at least two independent experiments with similar results.

Molecular Modeling—The spatial structure model of PKC-δ was constructed based on the known structure of protein kinases: PKC-θ, Protein Data Bank codes 1XJD, AKT2, 1O6L; and PKA, 1L3R, using the COMPOSER subroutine of SYBYL (Tripos, St. Louis, MO). Models of T507A and T507A/F500L mutants were made by creating point mutations in the sequence of the PKC-δ kinase domain reads and 15 residues, which are critical to maintaining an active conformation without phosphorylation: 1) residues in the activation loop itself; and 2) residues that directly contact the activation loop. Although no structure is available for the PKC-δ kinase domain, the similarity between PKC-δ and PKC-θ made it straightforward to find such residues in a “first-pass” homology model. The notable differences are in the activation loop itself and ~15 residues thereafter that are located in the C-lobe. Two Phe residues (Phe-500 and Phe-527) are notable among the few non-conservative changes (Fig. 1). Three attributes made them particularly interesting: 1) Phe is a very hydrophobic residue often involved in hydrophobic stacks that provide stable interactions. 2) They are located sufficiently close in the spatial model to make a hydrophobic stack between them plausible. 3) PKC-δ is the only PKC with a Phe at both positions, which could explain its uniqueness.

RESULTS

Two Phenylalanines In/Near the Activation Loop of PKC-δ Are Involved in Maintaining Kinase Activity—We sought a structural explanation for the dramatic difference shown previously between PKC-θ and -δ in their dependence on activation loop phosphorylation. We explored the possibility that sequence differences between PKC-δ and -θ, which are critical to maintaining an active conformation without phosphorylation are: 1) residues in the activation loop itself; and 2) residues that directly contact the activation loop. Although no structure is available for the PKC-δ kinase domain, the similarity between PKC-δ and PKC-θ made it straightforward to find such residues in a “first-pass” homology model. The notable differences are in the activation loop itself and ~15 residues thereafter that are located in the C-lobe. Two Phe residues (Phe-500 and Phe-527) are notable among the few non-conservative changes (Fig. 1).
As an initial test of whether these Phe were critical to activation loop independence of PKC-δ, functional studies were performed with mutant constructs. The two Phe residues were mutated to the corresponding residue in PKC-δ/H9258 (F500L/F527H); mutations were made both in the wild type (WT) PKC-δ/H9254 and PKC-δ/H9251 mutant whose activation loop is unable to be phosphorylated (T507A).

In vitro kinase assays demonstrated that mutation of either Phe (or both) dramatically decreased kinase activity of PKC-δ/H9254 T507A (Fig. 1B). These Phe were unusually important because mutation of other residues corresponding to non-conservative changes (I499M, A505T, and T526N) did not have any significant effect on kinase activity of PKC-δ/H9254 (Fig. 1B). When the same analysis was conducted with mutants on the WT background, the Phe mutation did not have any defect. Thus the two Phe are critical for kinase activity only when the activation loop is not phosphorylated.

The N-terminal Extension of the Kinase Domain Helps Maintain Kinase Activity by Interactions with the Activation Loop—Two considerations prompted us to consider the possibility that residues N-terminal to the kinase domain contribute to activation loop stabilization. (a) A mutant of PKC-θ in which the residues corresponding to Phe-500 and Phe-527 in PKC-δ (Leu-531 and His-558) are mutated to Phe is still not active without activation loop phosphorylation, suggesting requirements beyond the two Phe for activity. (b) Modest sequence similarity between PKC-δ/H9254 and the A-helix of PKA (Fig. 2A) suggested that PKC-δ/H9251 might have an A-helix. This would be relevant because the A-helix in PKA (“A-helix”) plays a role in stabilizing the kinase domain (18, 19). To test the possibility that the sequence N-terminal to the kinase domain plays a role in stabilizing PKC-δ, we made four PKC-δ/H9254 kinase domain-only constructs varying with respect to: 1) presence versus absence of 20 residues of the N-terminal sequence (aa 329–348) and 2) WT versus T507A. The in vitro kinase activity of T507A is markedly impaired in the absence of the N-terminal extension but close to normal with that extension (Fig. 2B). In contrast, the presence of a phosphorylatable activation loop (WT) allows PKC-δ to function normally without its N-terminal extension. Thus, the N-terminal extension, like the pair of Phe, is necessary for kinase activity only in the absence of activation loop phosphorylation.

Structural modeling was undertaken to determine a structural explanation for the experimental results with the above constructs. Our
model of the kinase domain per se was based on the solved structure of its closest paralog, PKC-θ (Protein Data Bank code 1XJD). Two elements that are important for a functional kinase but are missing from that structure were derived from other closely related kinases: part of the C-terminal extension (aa 619–633) from AKT2 (PDB code 106L) and the rest (aa 636–645) from PKA (PDB code 1L3R), and the A-helix (aa 320–340) from PKA (PDB 1L3R). An important feature of the model is molecular interactions of the A-helix with the “bottom” of the kinase domain (Fig. 3). Its interaction with the C-helix is virtually identical to that observed in PKA, based on the conservation of critical Arg on the C-helix and a Trp on the A-helix. An interesting difference, however, is observed in the remainder of its interactions. In PKA the A-helix has strong hydrophobic interactions with a major hydrophobic groove on the bottom of the C-lobe (18, 19). In contrast, in the PKC-δ model the A-helix does not interact directly with the C-lobe but rather is connected to the C-lobe indirectly via hydrophobic residues in a short “insertion” in the activation loop (i.e. in PKCs but not PKA, Fig. 1A).

Specifically, 1) Tyr-334 in the A-helix interacts with Phe-500 in the activation loop; and 2) Ile-499 in the activation loop interacts with Phe-527 in the C-lobe. Thus, the three structural elements found experimentally to be important for PKC-δ catalysis in the absence of activation loop phosphorylation (Phe-500, Phe-527, and A-helix) are all involved in this chain of hydrophobic interactions unique to PKC-δ. The surface features of these structural elements that create the hydrophobic interactions are depicted in supplemental Fig. 1. Molecular dynamics was used to investigate in more detail the position and function of critical phenylalanine residues Phe-500 and Phe-527. In the case of WT PKC-δ an unusual predominance of a single conformation was observed during multiple simulations, suggesting that this conformation is strongly favored. Preference of that conformation also was observed in models of T507A, but when Phe-500 is substituted by Leu the structure becomes more flexible mainly because of the loss of the tight interaction between Phe-500 and Tyr-334, indicating the special role of those aromatic residues in maintaining this stabilization of the activation loop. This favored conformation is controlled by distinctive interactions of Phe-500 with Tyr-334 and Phe-527 with Ile-499. Thus, the two Phe residues unique to PKC-δ appear to be essential to form that stable conformation. It is notable that the structural elements necessary for this stabilization (the pair of Phe residues and motif in N-terminal region) have been conserved in most vertebrates (mouse, rat, dog, xenopus, and even pufferfish).

Analysis of ATP binding (supplemental Fig. 2) shows that the double Phe mutant F500L/F527H has a ~2-fold increase in the $K_m$ for ATP, confirming the role of those Phe in optimizing kinase domain conformation. This alteration was less than the increase caused by the T507A mutation, which was ~4-fold. The T507A mutant also had altered lipid dependence for activation (supplemental Fig. 3) as previously described (14).

Product Inhibition of PKC-δ T507A with Some Substrates—Because the activation loop forms part of the peptide substrate binding surface, the presence or absence of phosphorylation on the activation loop of PKC-δ could potentially change its substrate specificity (14, 20). This would be important to know, both for interpretation of results on kinase...
activity, and for its potential relevance to the functional effects of regulated phosphorylation of PKC-δ in cells. To explore this possibility, we compared PKC-δ WT and T507A substrate specificity by positional scanning with degenerate peptide, which has proved highly informative in investigating kinase peptide specificity (17). T507A was virtually identical to WT as assessed by this method for substrate positions between P−7 and P+6 (supplemental Fig. 4).

As an alternative way to assess peptide specificity, we analyzed phosphorylation by PKC-δ WT, T507A, and the FF mutant (F500L/F527H as a control) of a panel of 96 proteomic peptides. Those peptides were chosen from genomic sequences not only for similarity to the consensus sequence of PKC substrates but also for diversity between peptides in exact sequence. Consequently, the set incorporates a diverse set of candidate PKC phosphorylation sites. Examination of the patterns shows that at a low peptide concentration (1 μM) the extent of phosphorylation of all peptides by T507A was very similar to WT (correlation coefficient = 0.94). But at a higher peptide concentration (10 μM) rather marked scatter became apparent (correlation coefficient = 0.56) (Fig. 4). The distribution of peptides suggested that many fell within a fairly linear distribution, but that there were a substantial subset of peptides for which phosphorylation by T507A was substantially less efficient. The specificity of the FF mutant protein showed no such deviation either at low or high peptide concentrations (correlation coefficients > = 0.96 at both concentrations).

To confirm and extend this unexpected observation, we preformed a more careful peptide titration with four potentially informative well phosphorylated peptides for which we had sufficient quantity for analysis (Fig. 5A). Two peptides have classic titration curves (D40 and PARIS, Fig. 4B, peptides 3 and 4) in which phosphorylation increased with increasing peptide concentration and plateaued around 10 μM; these peptides are the ones for which the screening assay indicated equivalent phosphorylation by WT and mutant. A very different pattern was observed with the other two peptides (MLK3 and diacylglycerol kinase-α), which the screening assay had suggested behave anomalously with T507A (Fig. 4B, peptides 1 and 2). Their phosphorylation at low peptide concentrations by WT and T507A was very similar, but their phosphorylation by WT and T507A differed significantly at a high peptide concentration. Instead of a plateau, phosphorylation by T507A decreased significantly at high peptide concentrations. The results suggest that inhibition of phosphorylation by PKC-δ T507A occurs with some peptide substrates at high concentrations either as a result of substrate inhibition or product inhibition. To begin to distinguish these two possibilities we analyzed phosphorylation as a function of incubation time (Fig. 5B) for the two most informative peptides: 1) the peptide showing greatest high-dose falloff (MLK3, Fig. 5A); and 2) one of the peptides showing no high-dose falloff (PARIS, Fig. 5A). Phosphorylation
of PARIS peptide increased as a function of time and was similar between T507A and WT, showing a regular time-dependent increase. In contrast, phosphorylation of MLK3 peptide by T507A was similar to that of WT at early time points but dropped off as the reaction continued. The progressive decrease in efficiency of T507A phosphorylation of MLK3 (but not of PARIS) was consistent with inhibition of T507A by the accumulating phospho-MLK3 product, the binding of which to the kinase domain would be facilitated by its phosphate group occupying the positive charge pocket left empty by the pseudo-dephosphorylation of the activation loop phosphorylation site (Thr-507).

To test this, we synthesized phosphorylated and non-phosphorylated “inhibitor” peptides corresponding to: 1) the peptide showing greatest high-dose falloff (MLK3, Fig. 5A); and 2) one of the peptides showing no high-dose falloff (PARIS, Fig. 5A). The ability of each peptide to inhibit was tested over a range of peptide concentrations on WT and T507A (Fig. 6). The inhibitor peptide designed from the well behaved substrate (PARIS) was unremarkable; it was an inefficient inhibitor for both proteins and the corresponding phosphopeptide was a somewhat poorer inhibitor consistent with its less favorable charge. The inhibitor peptide designed from the anomalous substrate (MLK3) behaved in a different manner. It was a much better inhibitor for both WT and T507A than unphosphorylated PARIS. Most notable is the finding that the MLK3 phosphopeptide showed a very strong phosphate-dependent inhibition primarily of T507A ($K_i \approx 2.5 \mu M$). These results indicated that unphosphorylated PKC-δ (T507A) was very susceptible to product inhibition by some of its substrates.

**PKC-δ T507A Is Defective in Reporter Activation in Cells**—The foregoing studies demonstrate that although the pseudo-dephosphorylated PKC-δ (T507A) has “normal” in vitro kinase activity on many substrates, it also has significant functional alterations of in vitro phosphorylation of some peptides and susceptibility to inactivation by other mutations. Because non-phosphorylated PKC-δ is present in cells, we addressed the critical question as to whether non-phosphorylated PKC-δ (T507A) functions normally in cells using NF-κB and AP-1 reporter assays. Those studies demonstrate that constitutively active PKC-δ is a potent activator of both NF-κB and AP-1 reporter constructs in Jurkat T cells (Fig. 7A). In contrast, the T507A mutant of the constitutively active PKC-δ failed to activate either NF-κB or AP-1 despite similar levels of expression. Because such reporter assays require kinase activity (sup-
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FIGURE 8. Substrate selective defect of PKC-δ T507A in phosphorylation in cells. A and B, a Lck-targeted kinase domain construct (WT or T507A) was transfected into HEK293T cells together with an HA-tagged substrate construct containing the PKC phosphorylation site from MARCKS (A) or CREB (B). After 18 h the cells were lysed. Phosphorylation of the substrate construct was assessed by Western blot with the corresponding phospho-specific antibody. The MARCKS substrate was phosphorylated only by WT PKC-δ (A), whereas the CREB substrate was phosphorylated by both (B). Expression of the substrate constructs (MARCKS or CREB) and the kinase domain constructs was assessed by Western blot with antibodies against peptide tags. Western blot results were quantitated and represented in a bar graph in which phosphorylation by WT is shown as 100% and phosphorylation by T507A was expressed as percent of the WT. Note that the CREB substrate undergoes a mobility shift after phosphorylation. The arrowhead indicates the position of a slightly lower mobility nonspecific band that is also seen in untransfected cells (not shown), C and D, PKC-δ WT or T507A protein was purified from transfected HEK293T cells, adjusted to equal protein concentrations by Western blot comparisons, and the immune complex kinase assay was done with serially diluted MARCKS peptide (C) or CREB peptide (D) as substrates.

Supplemental Fig. 5), the simplest interpretation is that unphosphorylated PKC-δ is unable to mediate phosphorylation of the specific sites in cells involved in NF-κB and AP-1 activation.

To exclude the possibility that activation loop phosphorylation may regulate localization of PKC-δ in the cells, which might be critical for its function in cells, we made Lck motif-targeted PKC-δ kinase domain constructs (PKC-δaa 329–676) with Thr-507 mutated or not. The Lck motif contains the N-terminal 15 amino acids of Lck, which has been shown to be the lipid modification site that targets Lck to membrane lipid rafts. The Lck motif-targeted PKC-δ kinase domain construct was capable of activating both NF-κB and AP-1 reporters (Fig. 7B). But constructs in which the Thr-507 was mutated totally lost that capability. Untargeted PKC-δ kinase domain had little activity in reporter assay (data not shown). Because both constructs were targeted by the same motif, the results indicated that kinase activity rather than localization was the reason the activation loop mutant failed to activate the reporters.

PKC-δ T507A Has a Substrate Selective Defect in Phosphorylating Substrate in Cells—Clarification of the molecular defect in T507A would be provided by an assay of its capacity to directly phosphorylate its substrate in cells; ideally this assay should be in the same cells used for the reporter assay. However, to our knowledge no physiologically relevant PKC-δ substrate has been definitively identified in T-cells. We therefore developed an in-cell assay that involves co-transfection of a kinase domain construct and a fusion protein that includes site(s) suitable for phosphorylation by PKC, such as those in MARCKS (21). Colocalization is a major physiological mechanism for facilitating signal transduction, including phosphorylation (22, 23). Therefore, this assay exploits colocalization by incorporating into both the kinase and substrate constructs an Lck targeting motif that localizes them to the same membrane compartment (see supplemental Fig. 6). To determine whether the in-cells defect of T507A observed in reporter assays reflects a defect in catalysis, we analyzed its ability to phosphorylate in this in-cells co-targeting assay. The results demonstrate a striking defect in its in-cells phosphorylation of the co-targeted MARCKS sites (Fig. 8A). We extended the analysis to a different co-targeted substrate that contained the Ser-133 of CREB-α, which is described to be a substrate for both PKA and PKC (24). In contrast to the results with the MARCKS sites, the CREB site was phosphorylated similarly by WT and T507A (Fig. 8B); phosphorylation of CREB peptide is detectable both by phosphorylation antibody and by a decrease in mobility on SDS-PAGE. Thus, the results indicate that PKC-δ T507A is catalytically active but has a substrate-selective defect in phosphorylating substrates in cells.

We hypothesized that the selective defect of T507A in cells might relate to the product inhibition of the selective substrate in vitro. So we tested PKC-δ WT and T507A activity on MARKCS and CREB peptide in in vitro kinase assays (Fig. 8, C and D). WT and T507A phosphorylate CREB peptide equally well. At low peptide concentrations, WT and T507A both phosphorylated MARCKS peptide equally well. But at high peptide concentrations, T507A activity is significantly lower than WT. So T507A-specific product inhibition correlates with the defect of phosphorylation in cells.

T507A in Apoptosis—Because PKC-δ has been demonstrated to mediate the biologically important process of apoptosis (25), we investigated functional activity of T507A in apoptosis. In cellular models of apoptosis, caspase cleavage of PKC-δ to generate an active catalytic fragment is often required for apoptosis (25, 26). We used a well characterized model of apoptosis induced by kinase domain transfection (26); our constructs correspond to the region produced by caspase cleavage. We found that PKC-δ kinase domain T507A caused apoptosis judged by nuclear DNA condensation and fragmentation (as well as rounding up of cells) (Fig. 9A). T507A-induced apoptosis was comparable with that observed with the WT PKC-δ kinase domain expressed at comparable levels. As shown previously (26), apoptosis depends on the catalytic activity and was not observed with the kinase-dead K378W mutant. Thus, for inducing apoptosis, activation loop-dephosphorylated PKC-δ was as effective as the WT, in sharp contrast with NF-κB and AP-1 reporter activation.

We considered whether apoptosis induction by the WT PKC-δ kinase domain might be mediated by a pool thereof that is not phosphorylated on Thr-507. Using in-cell treatment with phosphatase inhibitor, we observed that the amount of Thr-507 phosphorylation could be increased 6-fold within 20 min without a change in PKC-δ kinase domain expression (Fig. 9B). Thus, at least 85% of WT PKC-δ kinase domain is not phosphorylated on Thr-507. This unphosphorylated pool...
is about comparable with that present in the T507A transfection and therefore sufficient to explain the apoptosis observed. This raises the possibility that the Thr-507-phosphorylated PKC-δ kinase domain may not mediate apoptosis.

**DISCUSSION**

The foregoing studies were initiated to understand the structural basis and significance of the finding that the catalytic activity of PKC-δ is independent of activation loop phosphorylation, unlike all other PKCs. Three particularly notable aspects of the findings warrant discussion. First, PKC-δ has a unique structural mechanism for stabilizing its activation loop, which enables it to be catalytically active in the absence of activation loop phosphorylation. Second, the mutant PKC-δ lacking activation loop phosphorylation shows striking susceptibility to inhibition by certain of its phosphopeptide products. Third, PKC-δ T507A, which lacks activation loop phosphorylation, differs profoundly in cells from WT PKC-δ; it is defective in activity in two reporter assays, and displays a substrate-selective defect in phosphorylation when co-targeted with substrates in cells but it is as active as WT PKC-δ in induction of apoptosis.

The structural mechanism by which PKC-δ stabilizes its activation loop in the absence of phosphorylation is unique among AGC kinases. It involves hydrophobic interactions of the activation loop with both a short N-terminal α-helix and with an aromatic residue in the C-lobe. The N-terminal α-helix of the PKC-δ kinase domain has fundamental similarities to the α-helix of PKA both in terms of its general position and of the detailed mechanism of the α-helix–stabilizing interaction with the C-helix. All novel and classical PKCs have the Arg at the C-terminal of the C-helix that PKA uses for binding to the A-helix. However, only PKC-δ has the corresponding Trp in a suitable position to contribute to binding of an A-helix in the manner used by PKA. Despite the similarity, unlike PKA, the A-helix of PKC-δ binds directly to the activation loop through hydrophobic interactions (see “Results” and supplementary figures). Phe-500 plays a critical role as the principal contact between the activation loop and A-helix. Molecular modeling of F500L mutations shows that it is critical to maintenance of activation loop backbone conformation; i.e. conformation changed in T507A/F500L but not in T507A (supplementary Fig. 7). The strategy of an N-terminal extension stabilizing activation loop conformation has been observed in only one other kinase, casein kinase II (CKII) (27), which does not require activation loop phosphorylation for activity and prefers highly acidic protein substrates. The isolated catalytic subunit of CKII is constitutively active. But when interacting residues in the N-terminal extension or activation loop are deleted or mutated, CKII loses activity or its activity become dependent on interaction with the regulatory subunit (28, 29). The structural basis of stabilization of PKC-δ and CKII involves the same three discrete regions and are fundamentally similar. Aromatic residues are present in all three regions: N terminus, activation loop, and beginning of the F-helix. Multiple aromatic stacks provide stable contact of the N terminus to the activation loop and of the activation loop to the F-helix; the end result is an active conformation of the activation loop. Thus, PKC-δ utilizes an unusual mode of activation loop stabilization that has only been observed in one other context in a distantly related kinase.

Activation loop phosphorylation has been demonstrated to have several possible functional consequences for kinases (5, 7, 8, 30). One paradigm for the function of phosphorylation is “gated activation loops” in which phosphorylation prevents the activation loop from assuming a conformation that blocks substrate access (9). Another more widely relevant paradigm is that phosphorylation stabilizes the activation loop in an optimal conformation that facilitates phosphoryl transfer (9). There are a few citations that suggest an additional paradigm in which activation loop phosphorylation alters substrate specificity. In one instance the activation loop phosphate of CDK2 was shown to be important for substrate binding/alignment via its interaction with the P+3 lysine (31). In contrast, two other reports of altered specificity cannot be explained on such a clearly defined structural basis. One shows that activation loop phosphorylation of calcium/calmodulin-dependent kinase I is not simply an on/off switch but broadens the range of phosphorylated substrates (32); analysis of kinetic parameters demonstrated a 40-fold improvement in binding. Steinberg and colleagues (14) demonstrated that PKC-δ from activated cells was able to phosphoryl-
Regulatory Role of PKC-δ Thr-507 Phosphorylation

| High Dose Inhibition with PKC-δeTA507A? | protein name | Sequence | Source |
|----------------------------------------|--------------|----------|--------|
| No                                     | D40          | GPL\xE5PLSSK | RRK-S-RLK | Fig 5 |
|                                        | PARIS        | EY        | RRA-S-RRAV | Fig 5 |
|                                        | CREB         | EI        | RRP-S-YRKILD | Fig 8 |
| Yes                                    | MLK3         | I/VRK\x2012PHR | RRG-T-FKRS | Fig 5 |
|                                        | DGK iota     | NR        | KRT-S-FKRKA | Fig 5 |
|                                        | MARCKS       | W         | KRP-S-FKSKFKL | Fig 8 |

Table 1. Tabulation of peptide sequences and functional properties

We suggest the possibility that the unique design of PKC-δ among PKCs (i.e. activity without activation loop phosphorylation) evolved due to the combination of: 1) the PKC-δ catalytic fragment evolving an important function (e.g. in apoptosis); and 2) the vulnerability of catalytic fragments to activation loop dephosphorylation. If induction of apoptosis by the caspase-cleaved kinase domain depended on Thr-507 phosphorylation, it would be quite vulnerable to inactivation by dephosphorylation because the regulatory domain has been shown previously to help protect against PKC activation loop dephosphorylation (34). This vulnerability is borne out in our studies in which the catalytic fragment is poorly phosphorylated in cells (Fig. 9B), unlike full-length PKC-δ in the same cells whose Thr-507 phosphorylation in cells is not augmented by phosphatase inhibitor (supplementary Fig. 8). Evolution may have fixed this vulnerability by engineering phosphorylation independence into the kinase domain of PKC-δ.

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