A member of the novel protein kinase C (PKC) subfamily, PKCθ, is an essential component of the T cell synapse and is required for optimal T cell activation and interleukin-2 production. Selective involvement of PKCθ in TCR signaling makes this enzyme an attractive therapeutic target in T cell-mediated disease processes. In this report we describe the crystal structure of the catalytic domain of PKCθ at 2.0-Å resolution. Human recombinant PKCθ kinase domain was expressed in bacteria as catalytically active phosphorylated enzyme and co-crystallized with its subnanomolar, ATP site inhibitor staurosporine. The structure follows the classic bipartite kinase fold and shows the enzyme in its active conformation and phosphorylated state. Inhibitory interactions between conserved features of staurosporine and the ATP-binding cleft are accompanied by closing of the glycine-rich loop, which also maintains an inhibitory arrangement by blocking the phosphate recognition subsite. The two major phosphorylation sites, Thr-538 in the activation loop and Ser-695 in the hydrophobic motif, are both occupied in the structure, playing key roles in stabilizing active conformation of the enzyme and indicative of PKCθ autocatalytic phosphorylation and activation during bacterial expression. The PKCθ-staurosporine complex represents the first kinase domain crystal structure of any PKC isotypes to be determined and as such should provide valuable insight into PKC specificity and into rational drug design strategies for PKCθ selective leads.

Inhibitors of PKCθ are currently being used in clinical trials for various types of cancer, and a PKCθ inhibitor is being used in clinical trials for diabetes-related retinopathy (1). PKC and PKB/AKT kinase domains are related by sequence homology; however, there are key structural differences in the regulatory domains and second messenger cofactor requirements. PKB/AKT contains an N-terminal pleckstrin homology domain regulated by phosphoinositide second messengers, a central catalytic kinase domain, and a C-terminal regulatory region facilitating key protein-protein interactions with signaling molecules like Src kinase (2). PKC kinases can be regulated by calcium, diacylglycerol, and phorbol esters and are divided into three subfamilies based on their cofactor requirements (3): conventional (PKCa, PKCb, PKCα), novel (PKCγ, PKCe, PKCθ, PKCζ), and atypical (PKCδ, PKCδa, PKCδb, PKCδc) isoforms. PKC kinases have a C-terminal catalytic kinase domain and an N-terminal regulatory co-factor-binding domain. The N-terminal motifs comprise the phosphatidylinositol- and diacylglycerol-binding C1 motifs and calcium-binding C2 domain, in addition to a pseudosubstrate sequence motif that is regulated by cofactor binding.

The closely related PKC isoforms have been shown to have important roles in T cells (PKCa, PKCb, PKCα), B cells and mast cells (PKCb, PKCδ), and macrophages (PKCe), contributing to adaptive and innate immunity (3). Both PKCθ and PKB/AKT are implicated in T cell signaling leading to T cell activation and survival (4–6). However, the expression and role of PKCθ are relatively restricted to T cells, with signaling in response to TCR stimulation contributing to T cell activation and cytokine production (7–9). PKCθ co-localizes to the immunological synapse in response to T cell activation (10). Thus, PKCθ inhibition is potentially desirable in T cell leukemias (11) and T cell-mediated allergic and autoimmune disorders.

Among AGC superfamily kinases, the kinase domain crystal structures have been determined for both PKB/AKT- and cAMP-dependent PKA (12) but not for a PKC isoform. The homologies in the kinase domain ATP-binding site have been a challenge in the development of highly specific inhibitors as disease therapies (1, 13). Structural elucidation of kinase active sites and comparison with that of closely related family members greatly increases our understanding of the mechanism of enzyme action and divulges issues regarding selectivity. A Rho kinase (AGC superfamily) inhibitor Fasudil/HA1077/1-(5-isoquinolinesulphonyl)homopiperazine HCl, belonging to the isoquinoline sulfonamide class of compounds, also inhibits both PKA and PKC in a reversible and ATP competitive manner (14). This kinase inhibitor is a therapeutic drug in treating cerebral vasospasm and has recently been co-crystallized with the PKA catalytic subunit to define key interactions of the kinase inhibitor within the ATP binding site (15).

The crystal structure of PKA revealed that the invariant amino acids in the highly conserved kinase catalytic core are clustered at the sites of nucleotide binding and catalysis (13). The PKB/AKT active enzyme structure complexed with AMP-PNP and substrate peptide revealed mechanistic implications of key phosphorylations of the kinase domain (16). More re-
cently, the comparison of the PKB/AKT structure with PKA structure (17) provided explanations for distinct substrate specificities of the similar active kinase domain conformations. Yang et al. (16) have also activated potential PKC, SGK, p70, and p90 S6 kinase substrate interactions based on homologies with the GSK-3 substrate binding residues of PKB/AKT. Till now these mechanistic insights remained to be confirmed by a PKC crystal structure, and the studies presented here attempt to define PKC kinase domain characteristics. We report the x-ray structure elucidation of the PKCθ catalytic domain, with mechanistic insights into similarities and distinctions from the closely related PKB/AKT and PKC catalytic domains. In addition, the structural information of the staurosporine-complexed PKCθ kinase domain presented here will aid in the rational design and optimization of selective small molecule inhibitors for therapeutic use for inhibiting PKCθ in specific targeting of T cells.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification**—The C-terminal catalytic domain of PKCθ (residues 362–706) was cloned into a PET-18b expression vector. This vector introduced a hexahistidine tag to the C terminus of the expressed protein, and a methionine-glycine amino acid pair was introduced by site-directed mutagenesis. Expression and Purification—Transformation of Bl21 (DE3) for overexpression. A 10-liter cell strain BL21-DE3 for overexpression. A 10-liter cell culture was expanded at 37 °C to an A600 of about 0.4. The temperature was then lowered to 25 °C before addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.1 mM to induce expression. The cells were grown for an additional 4 h before they were harvested.

Harvested cells were resuspended in 25 mM Tris, pH 8.0, 25 mM NaCl, 5 mM 2-mercaptoethanol, 5 mM imidazole, 50 μM ATP, and protease inhibitors and lysed using a microfluidizer. The lysate was applied to 20 ml of nickel-nitrilotriacetic acid resin for 1 h at 4 °C. The resin was subsequently poured as a chromatography column and washed extensively with the same buffer including 25 mM imidazole.

Gene-bound to the resin was eluted with 200 mM imidazole buffer and then immediately loaded onto an anion exchanger HQ. The column was washed with 25 mM Tris, pH 8.0, 25 mM NaCl, 5 mM DTT, 50 μM ATP before being resolved by the application of a linear gradient from 25 to 500 mM NaCl. Fractions containing PKCθ were selected by SDS-PAGE, pooled, and diluted 2-fold with 25 mM Tris, pH 8.0, 5 mM DTT and loaded onto a heparin chromatography column. The flow-through was applied to a hydroxypatite column and washed extensively with 25 mM Tris, pH 8.0, 50 mM NaCl, 5 mM DTT. A linear gradient of sodium phosphate from 0 to 100 mM eluted the target protein. The protein was then sized as a monomer on a Superdex 200 size exclusion chromatography column. The purified protein was dialyzed overnight against 25 mM Tris, pH 8.0, 50 mM NaCl, 5 mM DTT and concentrated to 7 mg/ml (determined by the Bradford assay) before being used for crystallization experiments.

**Kinase Assays and Data Analysis**—ATP, ADP, phosphoenolpyruvate, NADH, pyruvate kinase/lactate dehydrogenase enzymes, staurosporine, acetonitrile, and the buffer HEPEs were purchased from Sigma. Peptide substrate was purchased from AnaSpec (San Jose, CA), Syn Pep (Dublin, CA), or Open Biosystems (Huntsville, AL). The enzymatic activity of the protein was determined using the coupled pyruvate kinase lactate dehydrogenase assay followed spectrophotometrically at 340 nm. The standard reaction, except where indicated, was carried out in 25 mM HEPEs, pH 7.5, 10 mM MgCl2, and 2 mM DTT, 0.008% Triton, 100 μM NaCl, 20 units of pyruvate kinase, 30 units of lactate dehydrogenase, 0.25 mM NADH, 2 mM phosphoenolpyruvate. The PKCθ concentration was in the range of 0.156–0.312 μg/ml. The kinetic analysis was carried out in a 384-well plate at 25 °C on a Molecular Devices spectrophotometer in a final volume of 0.080 ml. The steady state kinetic parameters were determined in the buffer described above containing varied sucrose or Ficoll 400. Data were fitted to Equation 1 for normal Michaelis-Menten kinetics,

\[
\nu = \frac{V_{max}[S]}{K_m + [S]} \quad \text{(Eq. 1)}
\]

where [S] is the substrate, \(V_{max}\) is the maximum enzyme velocity, and \(K_m\) is the Michaelis constant. For inhibition kinetics the data were fitted to a competitive inhibition model in Equation 2,

\[
\nu = \frac{V_{max}[S]/K_c(1 + [I]/K_i) + [S]}{K_{in}} \quad \text{(Eq. 2)}
\]

where \(K_{in}\) is the slope inhibition constant. The data were analyzed using Sigma Plot 2000 Enzyme Kinetics Module from SPSS Science (Richmond, CA).

**Crystallization and Structure Determination**—Crystals with staurosporine were obtained at 18 °C from hanging drops containing 1 μl of protein/staurosporine solution (at ~1:1 molar ratio) and 1 μl of precipitating solution (2 M ammonium sulfate, 40 mM DTT, and 0.1 M bis-tris, pH 5.0). The crystals belong to the monoclinic space group C2, with one protein-staurosporine complex in crystallographic asymmetric unit. Prior to data collection, crystals were stabilized in solution containing mother liquor plus 25% glycerol and flash-frozen in a 100 K nitrogen stream. The x-ray diffraction data were collected to 2-Å resolution at the Advanced Light Source (Berkeley, CA) using a Quantum-4 CCD detector (Area Detector Systems) and then reduced and scaled with HKL-2000 (18). The structure was solved by molecular replacement with AMORE (19) using the structure of PKA (PDB code: 1STC (20), in which the A-helix, glycine-rich loop, activation loop, and C-terminal tail were omitted from the search model. The rotation and translation function solutions were found using data from 8 to 3.5 Å. The BUSTER

**TABLE I**

| Statistics for data collection and refinement |
|----------------------------------------------|
| **Data collection**                           |
| Space group                                  | C2 |
| Unit cell dimensions (Å)                     | \(a = 139.6, b = 42.4, c = 67.7, \beta = 116.2°\) |
| Resolution range (Å)                         | 20.0–2.0 |
| Completeness (%)                             | 94.7 |
| Unique reflections                           | 23,134 (1,520) |
| Average Ia(Io)                               | 19.3 (2.5) |
| \(R_{free} (%)\)                             | 4.1 (23.6) |
| **Model refinement**                         |                                             |
| Maximum resolution (Å)                       | 2.0 |
| Number of reflections (free)                 | 21,992 (818) |
| \(R_{work}/R_{free}\)                       | 20.1/21.6 |
| No. of protein atoms                         | 2,353 |
| No. of waters                                | 115 |
| Root mean square deviations                  | 0.005 |
| Bonds (Å)                                    | 1.082 |

\(a R_{sym} = \Sigma I_{hkl} - (I_{hkl})/\Sigma I_{hkl}\), where \((I_{hkl})\) is the average intensity over symmetry equivalents. Numbers in parentheses reflect statistics for the last resolution shell (2.07–2.0 Å).

\(b R_{free} = \Sigma [F_{obs} - |F_{calc}|]/\Sigma F_{obs}\), where \(R_{free}\) is equivalent to \(R_{work}\), but calculated for a randomly chosen 4% of reflections omitted from the refinement process.

**TABLE II**

| Steady state kinetic parameters for PKCθ |
|------------------------------------------|
| **Varied substrate**                     |
| \(K_{c(app)}\)                            | \(k_{cat}\) |
| \(K_{cat}\)                               | \(K_{m}\) |
| **μM**                                   | s⁻¹ | μM⁻¹ s⁻¹ |
| FARKGSLRQ                                | 6.5 ± 0.8 | 18 ± 1 | 2,700,000 |
| ATP                                      | 49 ± 5   | 18 ± 1 | 360,000 |

**Inhibition constants for PKCθ**

| Substrate | Inhibitor | Inhibition pattern | Inhibition constant |
|-----------|-----------|--------------------|---------------------|
| ATP       | Staurosporine | Competitive | \(K_{in} = 0.33 ± 0.04\) μM |
program (21) with TNT (22) were applied in generating maximum entropy omit maps to overcome model bias and to produce a more detailed map for the bound inhibitor. Several rounds of rebuilding (QUANTA, Molecular Simulations, Inc.) and refinement were performed. To further reduce model bias and to generate better maps, an “average map” was calculated using CNS (23) by overlapping seven protein kinase coordinates including those of PKA. The resulting electron density maps were of better quality, especially for loop regions in the N-terminal lobe (N-lobe). The model was further rebuilt and refined, and the quality of the model was judged by the decrease in $R$-factors. Refinement converged after many rebuilding cycles to an $R$-factor of 0.201 and $R_{\text{free}}$ of 0.216. Crystallographic data collection and refinement statistics are summarized in Table I. The final model contains protein residues 377–649 and 688–696, two phosphate groups attached at Thr-538 and Ser-695, one staurosporine molecule, and 115 water molecules. Residues 362–376 from the N terminus, C-terminal region 650–687, and residues 697–706 at the very C terminus were not detected in the electron density maps due to disordering. Structural figures were generated using PyMOL (24) and QUANTA (Molecular Simulations, Inc.).

Modeling of Peptide Substrates Bound to PKCα—The PKCα-staurosporine complex structure was aligned to a structure of PKB-β (AKT-2) in complex with a GSK-3 peptide (16), PDB code: 106L) via automatic alignment (using weights of 1.0 for both sequence homology and structural homology) in the Protein Design module of Quanta (Accelrys (2004), San Deigo, CA). The alignment was further adjusted manually to improve the overlap of the “hinge region” backbone (residues 459–461 in PKCα) between the two structures. The initial positioning in the PKCα structure of the ATP analog AMP-PNP in the ATP binding site, the peptide in its binding site, and the glycine-rich loop was determined by the alignment. The GSK-3 peptide was mutated to the PKCα activation segment (532GDAKTNTFCG541) peptide in one case and the hydrophobic motif peptide (689NMFRNFSFMN698) in the other case. The conformation and position of residues 386–395 (the glycine-rich loop plus two residues on either side) was taken from the PKB-β structure. First, the attachment points for the loop, residues 386–388 and 696–698, were energy minimized keeping the remainder of the structure fixed. Next, the peptide, the glycine-rich loop, AMP-PNP, and the surrounding residues (with an atom within 8 Å) were minimized subject to decreasing harmonic constraints. Finally, the peptide was

**Fig. 1.** Alignment of kinase domain sequences for human PKC isoenzymes. Conserved and moderately conserved amino acid residues are highlighted. Negatively charged residues are shown in red, positively charged in blue, and neutral and hydrophobic in green; cysteines and prolines are shown in brown and yellow, respectively. Phosphorylation sites and structural motifs known to be important for kinase function are indicated.
RESULTS AND DISCUSSION

Analysis of Protein Construct and Overall Structure—The bacterially expressed PKC\(\theta\) kinase domain used for structure determination (residues 362–706) showed higher molecular weight than expected. Treatment by \(\lambda\)-phosphatase and subsequent molecular mass determination by electrospray ionization-mass spectrometry indicated that the protein is phosphorylated at either six or seven amino acid residues (roughly a 50:50 mixture). The purified PKC\(\theta\) kinase domain was shown to have a higher specific activity than the full-length, commercially available PKC\(\theta\) (20). Phosphorylated sites in PKA are also shown. Structures were aligned using the central helices from the C-lobe. Both staurosporine-bound kinases display intermediate lobe structures with conformational differences in the glycine-rich loop.

Catalytic key residues (Lys-409, Asp-504, and Asp-522), invariant in all protein kinases, preserve intramolecular interactions observed in active kinase structures, in accordance with the structural criteria used to define catalytically active kinase conformations (12). As in most Ser/Thr kinase structures reflecting active enzymes (28), helix \(\alpha C\) (residues 421–437) is properly aligned for substrate binding and catalysis, and the activation loop (residues 522–544) bearing the essential phosphates, is mostly helical consisting of eight \(\alpha\)-helices (\(\alpha D\)–\(\alpha K\)). The ATP-binding site, occupied by staurosporine, with the adjacent peptide-substrate binding site open to solvent, constitute the active site cleft at the interface of the two lobes. The glycine-rich phosphate-binding loop (GXGXXG), which shows a broad range of conformations (12), including multiple conformations observed within a single crystal (15), connects the \(\beta 1\) and \(\beta 2\) strands (residues 386–394) and adopts a fixed and closed conformation.

The C-terminal hydrophobic motif (HM) FXXFS\(^*\) (residues 691–695), another conserved feature across AGC family, is adjacent to the hydrophobic groove of the N-lobe, in a location similar to the FXXF-binding pocket in PKA and PKB. Like in PKB, but in contrast to PKA that terminates with the FXXF sequence, HM in PKC\(\theta\) contains phosphoserine at position 695 (Ser-695(P)), a phosphorylation that substantially effects the enzyme crystallized in its phosphorylated state and in an active conformation.

As outlined in the Introduction, the kinase domains of PKA, PKB/AKT, and PKC are highly homologous. Within the PKC subfamily, isozymes display more than 60% sequence identity in the kinase domain and share three conserved phosphorylation motifs (Fig. 1). The overall fold of the catalytic domain of PKC\(\theta\) is very close to other protein kinase structures solved, with most similarities to those of PKB/AKT and PKA (Fig. 2). The conserved core of the structure is made of a small N-terminal lobe (residues 377–461) and a large C-terminal lobe (C-lobe) (residues 466–696), connected by a hinge linker (residues 462–465). The N-lobe is based on a five-stranded \(\beta\)-sheet (\(\beta 1\)–\(\beta 5\)) and two \(\alpha\)-helices (\(\alpha B\) and \(\alpha C\)), and the C-lobe is mostly helical consisting of eight \(\alpha\)-helices (\(\alpha D\)–\(\alpha K\)). The ATP-binding site, occupied by staurosporine, with the adjacent peptide-substrate binding site open to solvent, constitute the active site cleft at the interface of the two lobes. The glycine-rich phosphate-binding loop (GXGXXG), which shows a broad range of conformations (12), including multiple conformations observed within a single crystal (15), connects the \(\beta 1\) and \(\beta 2\) strands (residues 386–394) and adopts a fixed and closed conformation.

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PKC\(\theta\) has an additional conserved phosphorylation site referred to as the turn motif (residues 662–685 and Fig. 2). In the structure, a long polypeptide linker between the kinase domain and the C-terminal HM (residues 650–687) is disordered; therefore the region corresponding to the turn motif is not defined in the electron density maps. There is also no observable electron density for residues C-terminal to HM (residues 697–706). In both PKA and PKB, the corresponding C-terminal linker is structurally ordered extending across the ATP-bind-
Binding of Staurosporine—The natural broad-spectrum kinase inhibitor staurosporine, with micromolar potency against only few kinases and low nanomolar potency against most kinases, has been shown to have a higher degree of selectivity toward PKC kinases (as reviewed in Ref. 30). Our kinetics data on PKC\(\theta\) activity in the presence of staurosporine indicate it to be a strong, ATP competitive inhibitor with a \(K_i\) value of 0.33 nM (Table II). Associations maintaining this tight interaction are clearly and well defined by electron density (Fig. 3A) and will be described in comparison with the binding of staurosporine to PKA (PDB code: 1STC (20)).

As expected, these high affinity ligands bind to the related enzymes in a generally similar binding mode. Staurosporine resides in the ATP-binding pocket, forming four potential hydrogen bonds with the protein backbone (Fig. 3A) and extensive van der Waals contacts with the surrounding residues from both lobes and the hinge linker (Table III). Hydrogen bonding between the lactam ring of staurosporine (N-1, O-5) and the hinge polypeptide (O-459, N-461) is well reproduced in all protein kinase-staurosporine structures determined thus far. The remaining two interactions are from the glycosidic ring of the inhibitor and from Phe-508 of the C-lobe. The main chain carbonyl of this residue (O-508) bonds to the methymalino nitrogen (N-4) and to the methoxy oxygen (O-6) of staurosporine. The latter interaction is not observed in the PKA-staurosporine structure (20); instead an additional hydrogen bond to the methamino group (N-4) is provided by the side chain residue Glu-127. Other distinctive features in individual inhibitor-enzyme interactions are as summarized in Table III (contacts calculated within 4 Å). For example, relatively more staurosporine to main chain van der Waals contacts are observed in PKC\(\theta\) than in PKA (43.5 versus 36.5%), which is largely attributed to the atoms in the phosphate binding loop. In PKA, on the other hand, additional side chain contacts to the inhibitor are provided from the residue of the C-terminal tail (Phe-327), a region that is of high disorder in our structure.

Despite this and other differences, the total number of polar and van der Waals interactions between staurosporine and PKC\(\theta\) (4 polar and 85 van der Waals) is identical to the number calculated for staurosporine-PKA (Table III). In addition, the buried surface areas of staurosporine are comparable for the two enzymes (569 Å\(^2\) in PKC\(\theta\) and 563 Å\(^2\) in PKA, calculated using a solvent probe radius of 1.4 Å). It seems unlikely, therefore, that these rather subtle structural variations are the only factors that account for at least a 20-fold difference in inhibition and are sufficient to drive selectivity (staurosporine \(K_i\) against PKA \(\sim 8\) nM). Below, we analyze other structural features such as ligand-dependent conformational states of kinase domains that could be a contributing factor to inhibitor affinity/specificity.

The aligned structures of the PKC\(\theta\)- and PKA-staurosporine complexes display remarkably good agreement in overall conformation (Fig. 2B). Accordingly, comparative analysis with three so far reported main conformational states of protein kinases, “open,” “intermediate,” and “closed” (reviewed in Ref. 31) indicates that, except for the glycine loop, relative disposition of the N- and C-lobes in PKC\(\theta\) represents intermediate lobe structures (32), (20). In most intermediate kinase structures, including complexes with staurosporine, the glycine-rich loop also adopts a position that is intermediate between open and closed states (31). In contrast, and as represented in this crystal structure, the tip of the glycine-rich loop (residues 389–392) moves deep inside the phosphate-binding subsite, approaching staurosporine and assuming positions that would clash with the nucleotide phosphate moiety. As a result, two atoms of Ser-390 engage in hydrogen bonding with the metal binding residue Asp-522 (Asp-184 in PKA), and Phe-391 swings into the active site approaching the catalytic Lys-409 (Lys-72 in PKA) and helix \(\alpha\)C (Fig. 3B). These interactions shield the phosphate-binding site, promoting and stabilizing its closure so that the resulting conformation of the glycine-rich loop is even more closed than is apparent in PKA and other kinase complex structures (see legend to Fig. 3 and Ref. 31). Due to this, and in contrast to PKA, access to the residues that are crucial for...
catalysis is largely restricted. Consequently, on binding to PKCθ staurosporine appears to be able to exploit not only adaptational changes in lobe orientation but also changes in the glycine loop structure that ensure that the phosphate recognition site is effectively blocked.

The binary complex represented in this crystal structure highlights flexibilities of protein kinases to accommodate ligand-induced binding effects and offers an explanation of the higher inhibitory activity of staurosporine against PKCθ. Whether the binding mode of staurosporine to other PKC isoforms, at least to their active phosphorylated forms, employs the same combination of general and specific features as observed here for PKCθ remains to be seen. Further structural analysis of inhibitors complexed with PKC kinases in both active and inactive states is required to address the latter possibility.

**Activation Loop and Helix αC**—The activation loop and the central αC-helix share crucial roles in catalysis. The activation loop provides part of the binding surface for peptide substrates and together with helix αC also serves as a docking site for activating or inactivating co-factors (28). In most kinases these two highly variable structural regions fold into an active conformation as a consequence of phosphorylation on the activation loop (12). For PKCs, this is a single phosphorylation site at threonine located in the activation loop sequence between the invariant Asp-166 in PKA) and on the other, allow for the correct positioning of the catalytic base Asp-504 (Asp-165 in PKA), and on the other, allow for the correct positioning of the αC-helix, which is now properly aligned to place the catalytic base Asp-504 (Asp-166 in PKA), and on the other, allow for the correct positioning of the αC-helix.

As shown in Fig. 4A, all three oxygens of the essential phosphate Thr-538(P) are involved in hydrogen-bonding interactions, consistent with the results of the in vitro activity studies of the full-length PKCθ in which Thr-538 was substituted for glutamate (29). The T538E mutant has shown a 3-fold decrease in activity compared with the wild-type enzyme, indicating that the glutamic acid at this position would provide only a partial mimic of the phosphoamino acid. Thr-538(P) is placed beneath the αC-helix to compensate for the conserved cationic cluster formed by Arg-503 and Lys-409 (Arg-165 and Lys-72 in PKA). As has been shown previously, a K409R mutation eliminates the catalytic activity of PKCθ (11), confirming that this residue is essential for catalysis. In addition, and similar to PKA, Thr-538(P) hydrogen bonds with the side chain of the preceding Thr-536, an interaction that further tethers the phosphate ion to the activation loop and may help stabilize it.
Much of this networking is similar to the equivalent interactions in phosphorylated PKA and PKB. However, outside of this region, there are several features inherent to the PKC\(\theta\) structure and not found in either the PKA or PKB kinases. In both PKA and PKB, helix \(\alpha C\) presents a histidine side chain to contact the phosphate of threonine on the activation loop (His-87 in PKA and His-196 in PKB). In PKC\(\theta\), in a structurally equivalent position to His-87, at the tip of the helix, lies a strictly conserved cysteine residue, Cys-424 (Figs. 1 and 4). Because of this, Thr-538(P) is unable to form the equivalent, electrostatic or hydrogen-bonding, contact. Instead, there is an alternative double salt bridge interaction that links the \(\alpha C\) helix directly to the activation loop but does not engage the phosphate ion: from Arg-430, two turns along the helix, to

FIG. 5. Molecular surface of the HM-binding surface. The semitransparent molecular surface is in gray, ribbons of N-lobe are in cyan, and the C-lobe is in blue. The C-terminal HM segment shown in sticks adopts an extended conformation making extensive hydrophobic contacts with the hydrophobic channel in the N-lobe. Hydrogen-bonding contacts formed by Ser-695(P), backbone atoms of HM, and residues in the N-lobe (sticks) are indicated by dashed red lines. Insertion of Trp-436 in the HM-interacting surface is also shown. The inset on the left is a close-up view that depicts conformational differences at the C-terminal end of \(\alpha C\) helices in three different kinase structures: PKC\(\theta\) (blue), PKA (yellow), PKB (red), and PDK1 (green). Structure-based sequence alignment highlighting a single residue insertion that creates an additional helical turn in PKC\(\theta\) is also shown.

FIG. 6. A model of the peptide corresponding to the activation segment of PKC\(\theta\) (residues 532–541) and an ATP analog bound to the PKC\(\theta\) protein. The peptide and AMP-PNP are colored by element with carbons in cyan and shown as thick lines. The protein is colored by element with carbons in green, and Asp-465 is indicated by thick lines. The location of the P-3 pocket is labeled. Magnesium atoms are shown as purple spheres.
Glu-528 that sits just after Lys-527 and directly under Arg-430. Along with this, formation of an ion pair between Arg-430 and Glu-423, a helix capping interaction, appears to facilitate the correct orientation of the arginine chain and hence the anchoring of Glu-528 to the outer surface of helix αC (see also Fig. 5). Constellation of the three invariant residues of complementary charges, Glu-528, Arg-430, and Glu-423, placed at this inter-domain interface is unique to PKC kinases (Fig. 1) and as such is not shared by PKA or PKB. In structural terms, these newly formed, ~13 Å away from Thr-538(P), hydrogen-bonding interactions seem to accomplish the same role as a histidine-phosphothereonine contact in PKA and PKB by contributing to the correct positioning of both the αC helix and the activation loop. They may also have functional assignments specific for the PKC family, for example, by keeping interaction of the activation loop with the long αC helix fixed at this side while rendering easier access to the N-terminal side of the helix and Cys-424 therein, which may be required for substrate/effecter binding.

Further comparison with AGC kinase structures shows that the αC helix of PKCθ contains a single residue insertion that creates an additional helical turn at its C terminus and shortens the αC-β loop (see inset in Fig. 5). The first residue in the last turn, Trp-436, is largely exposed to solvent and forms part of the surface that faces the C-terminal HM (Fig. 5). While sequence analysis suggests that all PKC members will share a similar whole helical turn insert, a tryptophan insertion is not a conserved feature of this family (Fig. 1). Therefore, PKCθ may be unique in how this tryptophan residue specifically shapes and stabilizes the surface on which its own HM segments dock. In general, the insertion of an additional turn into the αC helix will impose conformational constraints on the flexibility of the αC-β loop and the mobility of the αC helix thereby helping to tether the critical helix in a productive position. As activation through orientation of the central αC helix is common to other protein kinases, it is possible that these additional conformational constraints, not observed in either of the AGC kinase structures, have enzyme-specific roles in the activation or regulation of PKC family.

Hydrophobic Motif—The HM phosphorylation of PKCθ was shown to be required for optimal enzyme activity, with a 5-fold reduction of kinase activity in the full-length PKCθ S695A mutant immunoprecipitated from transfected HEK293 cells (29). Crystallographic data on PKB indicate that the structural role of the hydrophobic phosphorylation site is to tighten intramolecular association between the HM and the N-lobe to align the αC-helix favorably for catalysis (16, 33). The nature of interactions between the C-terminal HM, including phosphoserine 695, and the hydrophobic groove of the N-lobe is preserved in PKCθ, indicating that phosphorylation at this site plays an important and similar role in the structure of PKCθ.

The molecular surface of the N-lobe shows a channel, which hosts three characteristic aromatic side chains of the HM motif, Phe-691, Phe-694, and Phe-696 (Fig. 5). The deeply buried rings form extensive hydrophobic contacts with the surrounding residues from the αB and αC helices and β4 and β5 strands. The equivalent phenylalanine residues in PKA and PKB have been shown essential for protein stability and catalytic activity (34, 41). There are also several hydrogen-bonding contacts extending on both sides of the channel and anchoring the backbone of the HM (residues 691–696) to the αC helix (Lys-429) and to the β4 strand (residues 447–449). Finally, the top of the channel is capped by the phosphate ion of Ser-695(P). The latter forms two hydrogen bonds with the invariant Gln-449 (Fig. 1) from the β5 strand, an observation consistent with analysis of PKCθ activity in vitro, which indicates that the glutamic acid at the phosphor acceptor position (S695E) has slightly reduced and not abolished activity (29). These results also correlate with the least conservation of this phosphorylation site, which is reflected in atypical PKC kinases that share glutamate as a phosphate mimic at this position (35).

Concluding Remarks—With regard to PKCθ phosphorylation, the results described here strongly suggest that phosphorylation of the PKCθ kinase domain in E. coli is autocatalytic and are further supported by our mutation analysis in which the catalytically defective PKCθ kinase domain mutant K409W expressed in E. coli was found unphosphorylated. This appears to be in accord with the previous finding that the kinase-dead full-length PKCθ mutant K409W is not phosphorylated in growing HEK293 cells (29). This study also demonstrated that the PKCθ mutant T538A had completely lost phosphorylation of its hydrophobic motif and, to a certain extent, of its turn motif, indicating that both motifs represent sites of autophosphorylation and that their phosphorylation is regulated by the activation loop (29).

Interestingly, in contrast to the majority of other PKC isoforms (Fig. 1), the sequences surrounding the two critical phosphorylation sites of PKCθ, Thr-538 (KTNT*F) and Ser-695 (RNFS*F), both contain a positively charged residue at position P-3 (Lys-535 and Arg-693, respectively), which is compatible with the preferred substrate sequence R/KXXT*/S/F recognized by PKC family (36). A model of the peptide corresponding to the activation segment of PKCθ (residues 532–541) and an ATP analog bound to PKCθ (Fig. 6) explains the preference for positively charged residue at the P-3 position in substrates. In the model, the P-3 Lys-535 of the peptide interacts with Asp-465 and the ribose group of ATP. Also, Thr-538 of the peptide is positioned to react with a phosphate group of ATP as expected. A model of the hydrophobic motif peptide and an ATP analog bound to PKCθ (not shown) show similar interactions.

The autophosphorylation model for the PKCθ kinase domain would be analogous to the model described for the catalytic subunit of PKA, in which the phosphates can all be introduced autocatalytically (37). On the other hand, recent results with other PKC isoforms (reviewed in Ref. 38) suggest that the in vivo phosphorylation reaction depends on the universal PKC upstream kinase PDK1 and that PDK1-mediated activation loop phosphorylation followed by autophosphorylation at the HM is a general regulatory mechanism for PKCs (35). Although physical association of PKCθ with PDK1 has been demonstrated (29), there is still a lot to be determined to indicate whether or not PDK1 is responsible for the constitutive phosphorylation of the activation loop of PKCθ or whether it is primarily regulated by autophosphorylation.

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REFERENCES
1. Cohen, P. (2002) Nat. Rev. Drug Discov. 1, 309–315
2. Jiang, T., and Qiu, Y. (2003) J. Biol. Chem. 278, 15789–15793
3. Tan, S. L., and Parker, P. J. (2003) Biochem. J. 376, 545–552
4. Arendt, C. W., Albrecht, B., Soos, T. J., and Littman, D. R. (2002) Curr. Opin. Immunol. 14, 323–330
5. Bauer, B., Krumbock, N., Fresser, F., Hochholdinger, F., Spitaler, M., Simm, A., Uiberall, F., Schraven, B., and Baier, G. (2001) J. Biol. Chem. 276, 31627–31634
6. Bauer, B., Krumbock, N., Ghaffari-Tabrizi, N., Kamperf, S., Villunger, A., Wildes, M., Haneieger, H., Utermann, G., Leitges, M., Uiberall, F., and Baier, G. (2000) EMBO J. 19, 3645–3654
7. Altman, A., and Villalba, M. (2003) Immunol. Rev. 192, 53–63
8. Fleischer, C., Kohler, K., Graubner, T., Tabriz, N. G., Lute, C., Maly, K., Leitges, M., and Baier, G. (2000) J. Exp. Med. 197, 1525–1535
9. Sun, Z., Arendt, C. W., Ellmeier, W., Schaeffer, E. M., Sunshine, M. J., Gandhi, L., Ann, J., Petraliak, D., Kupfer, A., Schwartzberg, P. L., and Littman, D. R. (2000) Nature 404, 492–497
10. Diaz-Flores, E., Silicco, M., Martinez, A. C., and Merida, I. (2003) J. Biol. Chem. 278, 29208–29215
11. Villalba, M., and Altman, A. (2002) Curr. Cancer Drug Targets 2, 125–137
