Structural Aspects of the Functional Modules in Human Protein Kinase-Cα Deduced From Comparative Analyses

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ABSTRACT Three-dimensional models of the five functional modules in human protein kinase Cα (PKCα) have been generated on the basis of known related structures. The catalytic region at the C-terminus of the sequence and the N-terminal auto-inhibitory pseudo-substrate have been modeled using the crystal structure complex of cAMP-dependent protein kinase (cAPK) and PKI peptide. While the N-terminal helix of the catalytic region of PKCα is predicted to be in a different location compared with cAPK, the C-terminal extension is modeled like that in the cAPK. The predicted permissive phosphorylation site of PKCα, Thr 497, is found to be entirely consistent with the mutagenesis studies. Basic Lys and Arg residues in the pseudo-substrate make several specific interactions with acidic residues in the catalytic region and may interact with the permissive phosphorylation site. Models of the two zinc-binding modules of PKCα are based on nuclear magnetic resonance and crystal structures of such modules in other PKC isoforms while the calcium phospholipid binding module (C2) is based on the crystal structure of a repeating unit in synaptotagmin I. Phorbol ester binding regions in zinc-binding modules and the calcium binding region in the C2 domain are similar to those in the basis structures. A hypothetical model of the relative positions of all five modules has the putative lipid binding ends of the C2 and the two zinc-binding domains pointing in the same direction and may serve as a basis for further experiments.

INTRODUCTION

Protein kinases C (PKC)1,2 are a calcium-phospholipid-dependent subfamily of serine/threonine-specific enzymes that mediate responses of eukaryotic cells to various external stimuli.3–7 PKCs play a regulatory role in biological processes such as carcinogenesis,8 memory,9 and development10 and represent an important class of signal transduction enzymes.11,12 Tumor-promoting phorbol esters activate PKC in vitro, which is of particular medicinal interest.13

Members of the PKC family can be activated in vitro by Ca2+, phospholipid, and diacylglycerol14,15 in a way that is distinct from other protein kinases. Hence PKC is activated by tyrosine kinase receptors in signal transduction cascades that produce diacylglycerol.6 Phosphorylation of PKC in vivo is necessary for subsequent PKC activation,16,17 and PKCα can be completely inactivated, in vitro, by treating it with a Ser/Thr-specific protein phosphatase.18

PKC consists of two functional regions: the amino-terminal region (1–335 residues), which plays a regulatory role, and the carboxy-terminal region (336–656), which is catalytic (Fig. 1). A segment at the N-terminus of the regulatory region is recognized by the catalytic region and acts as an inhibitory pseudo-substrate. The pseudo-substrate sequence is followed by two homologous zinc-binding domains (together referred to as the C1 domain), which are contiguous and have similar amino acid sequences. In some isoforms of PKC there is only one zinc-binding domain (e.g., PKCδ).20

The structure of the second zinc-binding domain in rodent PKCβ has been determined by nuclear magnetic resonance (NMR).20 Recently the crystal structure of the second zinc-binding domain of PKCβ in a complex with phorbol ester has been reported.21 The structure consists of two small β-sheets with a C-terminal helix. Phorbol 13-acetate binds in a gap formed between two β-strands at the tip of the module.21 In the α, β, and γ isoforms of PKC the zinc-binding domains are followed by a domain that mediates calcium-dependent enzymatic and lipid-interacting...
properties (C2 domain). This domain is homologous to and occurs twice in the cytoplasmic region of synaptotagmins, which are synaptic vesicle membrane proteins. The crystal structure of the first of these domains in synaptotagmin I has been reported in both calcium-bound and calcium-free forms. The structure is made up of an eight-stranded β-sandwich constructed around a conserved four-stranded motif. Calcium ions bind in a cleft formed between two surface loops located at the N- and C-termini of the domain. The catalytic region is made up of two lobes. The adenosine triphosphate (ATP) is largely accommodated in the smaller lobe while peptide recognition takes place in the interface between the two.

Human PKCa has all modules including the two zinc-binding domains in the regulatory region. In the absence of activators, such as diacylglycerol and phorbol ester, the regulatory region inhibits the kinase activity by binding in the active site of the catalytic region. The pseudo-substrate shows variability in sequence among PKCs, but some amino acids are conserved. The important difference between pseudo-substrates and the substrates is the presence of an Ala residue in pseudo-substrates for the phosphorylatable Ser or Thr in substrates (see Stabel and Parker for a list of pseudo-substrate sequences). Upon binding activators at the regulatory region a structural change may release the pseudo-substrate from the catalytic site, allowing access for the substrate. Binding studies by Pap et al. show that phosphatidylinositol 4,5-biphosphate (PI4,5P2) also binds to PKC. It has also been shown recently that PI3,4,5P3 can activate PKC.

The availability of high-resolution crystal structures of the catalytic regions of protein kinases provides an opportunity to model the tertiary structures of the expanding kinase family. Three-dimensional models of the catalytic core of smooth muscle myosin light-chain kinase, epidermal growth factor receptor, and cell cycle protein kinase p34 were found to be consistent with observed properties and mutational analysis. We have generated a three-dimensional model for the catalytic region of human PKCa using its amino acid sequence and the crystallographic structure of cyclic AMP-dependent protein kinase (cAPK). A comparison of the sequences of PKCa and CDKB was aligned with cAPK and CDK2 (Fig. 2), and this prediction was later found to be consistent with the analysis of Cazaubon et al. The pseudo-substrate prototype has been modeled at the binding site of PKC by extrapolating from the structure of an inhibitor peptide (PKI) bound with the cAPK. The MnATP was also modeled in PKC by extrapolating from the known ternary complex structure. The model provides a structural explanation for the specificity of PKCa for the pseudo-substrate. The amino acid sequence of the second zinc-binding domain of human PKCa is identical to that of the rat PKCB, for which the NMR structure is reported. We have modeled the first zinc-binding domain and its complex with phorbol ester on the known NMR and crystal structures. The phorbol ester/diacylglycerol binding site at the groove formed by two loops is conserved in both the first and second zinc-binding modules of PKCa. A model involving a symmetric arrangement of the two zinc-binding domains is proposed. The C2 domain was modeled on the crystal structure of synaptotagmin. Finally, we suggest a spatial arrangement of all functional modules (in the inactive state) that is largely directed by the constraints in length of the loops connecting the contiguous modules and the nature of surfaces of the modules.

While describing the model, human PKCa numbering is followed in this paper. However, in discussions on the catalytic region and pseudo-substrate, cAPK and PKI peptide numbering is given in brackets. In discussions of the model and crystal structure of the zinc-binding domains we include in brackets the numbering scheme of Hommel et al., and in the section on the C2 domain model the numbering scheme of Sutton et al. is indicated in brackets.

**MATERIALS AND METHODS**

**Comparative Modeling of Catalytic Region**

The first crystal structure of cAPK was reported at 2.7 Å resolution (corresponding to the Brookhaven Protein Data Bank entry ICPK). Subsequently higher resolution structures were reported. Coordinates of the binary peptide-enzyme (IAPM) and ternary peptide-MnATP-enzyme (IATP) complex structures became available in the Brookhaven Protein Data Bank. We used IATP as the basis structure, since certain local regions of IAPM are ill-defined in the electron density, and hence the coordinates of such regions are unavailable.

The sequences of cAPK and CDK2 were aligned on the basis of their structural features, using the program COMPARER. The sequence of PKCa was then aligned with cAPK and CDK2 (Fig. 2), and both structures were initially considered as a basis for building the model of PKCa, since using as many basis structures as possible results in a more accurate model. However, the sequence of PKCa is more closely related to cAPK (sequence identity: 39.6%) than to CDK2 (sequence identity: 27.2%), and there are a few sterically unfavorable regions such as non-prolyl cis peptide bonds in CDK2. Hence we modeled PKCa only on the cAPK. Subsequent to the completion of our modeling work, crystal structures of mitogen-activated protein kinase (MAP), twitchin kinase, casein kinase 1, phosphorylase kinase, CDK2-cyclinA complex, CDK2-ATP-in-
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PKCa numbering is shown at the left and right sides of the figure and the sequence is shown in ten residue blocks. The five modules that have been modeled are underlined and indicated below as: pseudo-substrate, zinc-binding domain one, zinc-binding domain two, calcium-dependent lipid-interacting domain, and catalytic region.

hibrator complexes and insulin receptor kinase. which is Tyr specific, have also appeared. However, all these kinases are more distant to PKCa than cAPK.

The suite of programs encoded in COMPOSER and incorporated in SYBYL (Tripos, St. Louis, MO) was used to generate the 3-D model of PKCa. The structures of the conserved regions of cAPK have been extrapolated to the equivalent regions of PKCa. The variable regions are modeled by identifying a suitable segment from a known structure in the data bank. A search is made for a segment having the desired number of residues and the proper end-to-end distances across the three “anchor” Cα at either side of the putative loop such that the loop can be fitted joining the contiguous conserved regions. A template matching approach to rank the candidate loops was also used. The best ranking loop with no short contacts with the rest of the protein is fitted using the ring-closure procedure of F. Eisenmenger (unpublished results). Sidechains are modeled either by extrapolating from the equivalent positions in the basis structure where appropriate or by using rules derived from the analysis of known protein structures.

Modeling of Calcium-Binding Lipid-Interacting Domain

The crystal structure of the first C2 domain of synaptotagmin, which has been defined at 1.9 Å resolution, has been used as a basis to model the C2 domain of PKCa. The high sequence identity (34%), and many conservative replacements allows construction of a reasonably accurate model. The modeling procedure is identical to that described above for the catalytic region. The available coordinate set of synaptotagmin is for a form free from calcium. However, Sutton et al. report that the position of a
Fig. 2. Alignment of sequences of cAPK (latp) and CDK2 (cdk2) on the basis of structural features using COMPARER.46,47 Structural features are represented in the notation of JOY.45 The catalytic region of human PKCa (pkca) is shown aligned on the basis of sequence alone. cAPK numbering (top) and PKCa numbering (bottom) are shown.

The water molecule corresponds to that of calcium in the metal-ion-bound structure. Sutton et al.26 further report that the only change between the calcium-bound and calcium-free forms of synaptotagmin crystalline structures is the position of the sidechain of Asp 232, which is equivalent to Asp 248 of PKCa. This sidechain is reoriented in the PKCa model to bring the carboxylates within the coordination sphere.
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Modeling Zinc-Binding Domains

The amino acid sequence corresponding to the second of the zinc-binding domains in rat PKCβ is identical to that of the human PKCa. Initially the NMR structure of this domain from rat PKCβ was used as a basis to model the structure of the first of the zinc-binding domains in human PKCa, using COM-POSER. Subsequently crystal structures of the second zinc-binding domain of mouse PKCβ both uncomplexed and complexed with phorbol ester have been reported. We have remodeled the first zinc-binding domain of PKCa but in the complexed form with phorbol ester on the basis of the crystal structure of the complex.
Energy Minimization

The COMPOSER-generated models were energy minimized in SYBYL using the AMBER force-field. During the initial cycles of energy minimization the backbone was kept rigid and sidechains alone were moved. Subsequently all atoms in the structure were allowed to move during minimization. This approach kept disturbance of the backbone structure to the minimum. Energy minimization was performed until all short contacts and inconsistencies in geometry were rectified. During the initial stages of minimization, the electrostatic term was not included as the main objective was to relieve steric clashes and to rectify bad geometry.

Modeling of Complexes

The binding of a pseudo-substrate sequence at the catalytic site of PKCa was modeled by using the structure of cAPK in the complexed form. Positions of sidechains at the recognition site in PKCa as well as in the peptide were manually adjusted using the interactive graphics FRODO to optimize the interactions between the peptide and the enzyme. MnATP in the catalytic domain and calcium in C2 domain were modeled in a similar way. Modeling of the positions of zinc in the first zinc-binding domain did not involve any modifications in the sidechain conformations, as all the cysteine and histidine residues that are involved in coordination are absolutely conserved. Interactions of the phorbol ester are almost entirely with the mainchain carbonyls and amides, and the positions of these polar groups are modeled directly on the crystal structure.

RESULTS AND DISCUSSION

Tertiary Fold of the Catalytic Region

The sequences of cAPK and CDK2 were aligned on the basis of their three-dimensional structures (Fig. 2). The percentage sequence identity is 28.2. The sequence of PKCo was then aligned with these known structures; the percentage sequence identities of PKCs with cAPK and CDK2 kinase are 39.6 and 27.2, respectively. Obtaining an accurate model of PKCo using the structure of cAPK alone is attractive since the percentage sequence identity between PKCo and cAPK is more than the “twilight zone” of < 25–35% proposed by Doolittle. Our model of the catalytic region has been generated by using cAPK structure alone as the basis (See Materials and Methods for details).

Structurally variable regions (SVRs) were defined where there are insertions/deletions and where Gly 214 in cAPK, which adopts a positive p conformation, is replaced by proline in PKCs. This SVR and the other four SVRs of PKCo were modeled on fragments selected from a database of end-to-end and anchor residue distances (Table I). For the loop 487-496 (190–196) of PKCo, which is 10 residues in length, the number of anchor residues and the root mean square deviation of fitting required of a candidate loop were varied to explore as many loops as possible. The compatibility of conformational features with the sequence was also considered. The SVR was finally modeled on a segment from rhinovirus coat protein (PDB code: 2RS1) as a p-hairpin. The main reason to choose this segment is that the amino acid substitution by PKCo residues in this region for the residues in rhinovirus coat protein structure resembled substitution patterns seen in a database of homologous structures. The structural template of this loop in the rhinovirus coat protein matched well with the PKCo sequence in the loop region. The Asp 491–Gly 492 (192–193) segment at the tip of the hairpin has been modeled as a p-turn with a positive p conformation at Gly 492 (193) (Fig. 3). The flanking hydrophobic residues Met 490 (190) and Val 493 (193A) are involved in hydrophobic-hydrophobic contacts in the p-strands forming a hairpin structure. The glycine-rich loop (around position 350) is involved in the nucleotide binding. This region is conserved with cAPK and is a part of p-strand-turn-p-strand structure in the nucleotide and peptide bound form of the porcine heart cAPK structure and the twitchin kinase structure. The intrasteric regulated form of twitchin kinase, where the catalytic region is mirrored by a peptide in the sequence, resembles porcine cAPK in this loop region.

The sequence of the core of the catalytic region of PKCo (residues 338–620) could be easily accommodated in the fold of cAPK (Fig. 4). However, the structure of cAPK is larger than other kinase domains in having an additional N-terminal helix, and a long loop over the surface at the C-terminus. If there is an N-terminal p-helix in PKCo (and in other family members), it is probably at positions 300–307 (ELRQKF---corresponding to residues 25–31, DFLKKWE, in the cAPK structure). In cAPK the buried sidechain of Phe 26 interacts with Ala 97 and Leu 162. If Leu 301 in PKCo occupies an equivalent pocket it will interact with Leu 393 (97) and Ile 459 (162). If residues 300–307 of PKCo correspond to the N-terminal helix in cAPK, then PKCo will have an insert of about 20 residues between this helix and the first p-strand (compared with cAPK). Existence of such a helix has also been predicted for the C subunit of Dictyostelium discoideum and as a conserved feature of most eukaryotic protein kinases. This region corresponds to the V3-variable region of PKCs (Fig. 1) and lies outside the conserved catalytic region; our model does not include an N-terminal helix.

The C-terminal 50 residues of cAPK are looped over the surface of both lobes of the catalytic region, making very few specific interactions. However, Phe 347 and Phe 350 are buried (Fig. 2). Two phenylalanine residues toward the C-terminus of PKCo
TABLE I. Modeling of Structurally Variable Regions (SVRs) in PKC-α

<table>
<thead>
<tr>
<th>SVRs of PKC-α (residue nos.)</th>
<th>Modelled on PDB code and the first residue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>395–398</td>
<td>256B A Glu 49</td>
</tr>
<tr>
<td>487–496</td>
<td>2RS1 Pro 174</td>
</tr>
<tr>
<td>513–514</td>
<td>4PFK Ala 237</td>
</tr>
<tr>
<td>623–625</td>
<td>1CA2 Phe 226</td>
</tr>
<tr>
<td>645–648</td>
<td>2RS1 Phe 70</td>
</tr>
</tbody>
</table>

(residues 653 and 656) are well conserved among different PKCs. In addition, residues that form hydrophobic pockets that accommodate these two phenylalanine residues in cAPK are conserved in PKCa (Table II). In the present model of PKCa, Phe 653 and Phe 656 occupy the equivalent positions to Phe 347 and Phe 350 in cAPK. Phe 350 is the last residue in cAPK, and Phe 656 is the last residue in our model of the catalytic region of PKCa; however, sequence conservation suggests that the next five residues 657–661 will also have a defined structure as part of the catalytic region.

Su et al.64 have shown that the C-terminus of PKC is functionally important. While the deletion of the 11 C-terminal residues from bovine PKCa did not appear to affect activity, the deletion of 15 or more residues drastically reduced catalytic activity. We suggest that this may disrupt the C-terminal structure. The C-terminus packs against the helix between 381 and 393, and this may be important in determining the position of the helix. This helix is close to the activation loop, and its correct positioning may be important in determining the structure of this loop. The corresponding helix in CDK2 (PSTAIRE) moves on activation of the kinase.42,53

Permissive Phosphorylation Site in PKCa

The crystal structures of cAPK and CDK2 have been reported in the phosphorylated (active) and unphosphorylated (inactive) forms, respectively. In these enzymes, phosphorylation at Thr 197 (cAPK numbering) is essential for their activity. The crystal structure of MAP kinase49 corresponds to the low activity form, in which no phosphorylation has occurred at Thr 183 and Tyr 185 (MAP kinase numbering; corresponding to 196 and 198 in cAPK), situated at the “hot lip” of a variable loop region (L12 loop). Phosphorylation at these positions takes place upon recognition by another kinase MEK that presumably involves a drastic conformational change at this loop, exposing Thr 183 and Tyr 185 for phosphorylation. Zhang et al.49 report that the conformation of the L12 loop in MAP kinase is completely different from the equivalent loops of cAPK and CDK2.

The structure-based alignment of these loop regions is problematic for various reasons: (1) the lengths and positions of these loops in the three enzymes are quite different, as seen in the structural superposition of cAPK and CDK2 (data not shown); (2) cAPK is in the phosphorylated form at Thr 197 and is complexed with an inhibitory peptide, whereas the other two structures have no permissive phosphorylation; (3) the electron density at the

![Fig. 3. Superposition of the loop regions containing the phosphorylation site. Gray, cAPK crystal structure; green, PKC model. The backbone atoms are shown for the two loops with sidechains of phosphorylated Thr 497 (blue) and Asp 491 of PKCa. The positions of Asp 491 and Gly 492 forming a β-turn in the PKCa model are indicated. Dotted lines denote hydrogen bonds. Figure prepared using SETOR.89](image-url)
Fig. 4. Ribbon representation of the model of the catalytic region of human PKCa. Helices are shown in green and β-strand regions in blue. The modeled peptide is shown in golden yellow, phosphorylated Thr 497 in pink, and Ala 25 of the pseudo-substrate in white. The helices (B–J; A is absent in our model) and β-strand regions (1–9) are marked using the notation followed by Knighton et al. for cAPK. Figure prepared using SETOR.

TABLE II. Residues Interacting With C-Terminal Phenylalanines in CAPK and PKC-α

<table>
<thead>
<tr>
<th>CAPK</th>
<th>PKC-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys 76*</td>
<td>Lys 372</td>
</tr>
<tr>
<td>Val 79*</td>
<td>Val 375</td>
</tr>
<tr>
<td>Val 80</td>
<td>Ile 376</td>
</tr>
<tr>
<td>Ile 85</td>
<td>Val 381</td>
</tr>
<tr>
<td>Thr 88</td>
<td>Thr 384</td>
</tr>
<tr>
<td>Leu 89</td>
<td>Met 385</td>
</tr>
<tr>
<td>Lys 92*</td>
<td>Lys 388</td>
</tr>
<tr>
<td>Ser 109</td>
<td>Cys 406</td>
</tr>
<tr>
<td>Leu 116*</td>
<td>Leu 413</td>
</tr>
<tr>
<td>Met 118*</td>
<td>Phe 415</td>
</tr>
</tbody>
</table>

1CAPK residues indicated by * are buried (side-chain accessibility less than 4%). Residues 85, 88, 89, and 92 are in the helical segment between 85 and 97; Residue 109 is in the β-strand formed between 106 and 111; residues 116 and 118 are in the β-strand between 115 and 120.

equivalent T-loop region of CDK2 is not of good quality, and hence the atomic positions in this region can change significantly in the higher resolution structure; and (4) the superposition of Cα atoms, as a means of assessing structural similarity between distantly related proteins, is unlikely to yield correct alignment, as structural features at residue positions rather than the precise relative atomic positions tend to be conserved. (Percent sequence identity between cAPK and CDK2 is about 30.)

To minimize the uncertainties arising from rigid-body superposition, we have aligned the sequences of cAPK and CDK2 on the basis of local three-dimensional structural features and relationships using COMPARER (Fig. 2). The intention is to arrive at the proper alignment for the kinase folds, particularly in the “anchor” regions flanking the phosphorylation site-containing loop. Structural features such as local backbone conformation, solvent accessibility class, and hydrogen bonding patterns involving sidechain atoms have been considered in the alignment. Interestingly, the residues at 194–197 of cAPK align with corresponding residues at 194–197 of CDK2, emphasizing the topological equivalent of the permissive phosphorylation site at Thr 197 (Fig. 2). As the coordinates of MAP kinase and most other kinases are not yet available to us,
An alignment of the sequence of PKCa has been performed with the structurally aligned cAPK and CDK2 kinase sequences. Knowledge about preferred amino acid substitutions in a given structural environment, as observed in families of aligned protein structures, has been used to arrive at an alignment between the sequence of PKCa and known kinase structures (Fig. 2). As can be seen in the alignment, the phosphorylation sites in cAPK and CDK2 kinase align with Thr 497 or PKCa. Indeed, a mutational analysis of Cazaubon and Parker mapped the loop containing this threonine residue as a potential phosphorylation region.

Amino acid sequences of 34 isoforms of PKC have been aligned using the program MALIGN (data not shown). The threonine in question (Thr 497 in PKC or Thr 197 in cAPK numbering) is absolutely conserved (indicated by * in Fig. 1), tempting us to suggest that this Thr is a potential phosphorylation site in PKCa. An independent analysis by mutagenesis is entirely consistent with this prediction.

Catalytic Region-Ligand Interactions

The high-resolution structure of the PKI inhibitory peptide in cAPK was used as a basis to model the pseudo-substrate sequence RFARKGALRQK (residues 19–29; Fig. 1) at the catalytic site of PKCa. There are many positively charged side-chains but no acidic side-chains in the sequence, thus complementing the catalytic site of PKCa, which is surrounded by many acidic side-chains from the two lobes in the catalytic region. The charge distribution at the surface of PKCa, depicted by the program GRASP (Fig. 5), shows that the lobe interface encompassing the peptide-binding site is highly negatively charged. The amino acid sequence of the pseudo-substrate peptide corresponding to the region 15–24 of PKI is RTGRRNAIHD; there is good sequence similarity between the PKI and the PKCa pseudo-substrate sequence. Indeed, there is some cross-reactivity between the two. The PKI peptide adopts a helical conformation followed by a turn and an extended strand in the complex. Specific residues in this extended strand have been identified to recognize the catalytic site of cAPK. The region of PKI that is similar to the pseudo-substrate of PKCa corresponds to the turn-extended strand region. Ala 25 in the pseudo-substrate sequence corresponds to Ser or Thr that is phosphorylated in the substrates of PKC. The last residue in the pseudo-substrate sequence, a positively charged lysine, is modeled to interact with the phosphorylated Thr 497, which is negatively charged. An independent modeling and mutagenesis study demonstrates the importance of negative charge at this residue in the activation loop. It has been shown that mutation of Thr (phosphorylation site) in PKCβII to Glu, an acidic residue, results in catalytically active enzyme, and the mutant with this Thr replaced by Val, a neutral and non-phosphorylatable residue, results in an inactive enzyme. Indeed, it has also been shown that conventional and nonconventional activators produce similar structural alteration in PKCβII involving exposure of basic residues in pseudo-substrate that are otherwise shielded by the acidic residues in the enzyme.

The pseudo-substrate is accommodated comfortably in the catalytic site without any steric clash. Most of the side-chains that interact with the peptide in the catalytic site of PKCa are smaller in size than the corresponding residues in cAPK (Table IIIA), giving a net loss in volume of PKCa of 135 Å³. Two of the side-chains in the pseudo-substrate are bigger than the corresponding side-chains in the PKI peptide in the cAPK complex structure (Table IIIB). The net gain in volume in the pseudo-substrate of PKCa is 59 Å³. Thus the net loss in volume at the binding site in the PKCα-pseudo-substrate complex compared with the cAPK-PKI complex is 76 Å³. This might result in a closer arrangement of the two lobes in PKCα compared with those in cAPK.

There are many stabilizing interactions between the peptide and PKCa (Fig. 6, Table IV). Ala 25, which replaces a phosphorylation site in the substrate, is in hydrophobic contact with Phe 350.
and Met 484 (187). Phe 350 is conserved in most PKCs and conservatively replaced by Tyr in a few (indicated by * in Fig. 1). Met 484 is conserved in all but one of the isoforms, where it is replaced by Leu, which is also hydrophobic. Ala 25 is far from the permissive phosphorylation site at 497. Pears et al.\(^{29}\) reported that the replacement of Ala 25 by Glu results in a significantly increased effector-independent kinase activity. The increased activity of the mutant is likely to be due to a disruption in the complex structure. C\(^{9}\) of Ala 25 in the wild type is situated at about 4 Å from the sidechain of Asp 463 (166). Replacement of Ala 25 by Glu means a longer and negatively charged sidechain at 25 that results in a repulsive interaction with Asp 463, hence disrupting the complex structure, exposing the catalytic site in the enzyme.

The amino acid sequence of the pseudo-substrate RFARKGALRQK (residue numbers 19–29 in PKC numbering), the residues Arg 22, Arg 27, and Lys 29 are conserved as positively charged amino acids in most isoforms of PKC\(^{11}\) (Fig. 1). All arginines interact with acidic groups. Thus, Arg 22 is involved in extensive interaction with the sidechains of Asp 467 and Asp 424 (Table IV). Arg 27 is involved in hydrogen bonding with Asp 380. NZ of Lys 29, which is an extra residue compared with PKI, forms a hydrogen bond with one of the oxygens at the phosphate group on the phosphorylated Thr 497. All these sidechains in the enzyme (Asp 467, Asp 424, Asp 380, and Thr 497) are absolutely conserved in all isoforms of PKC. The conserved acidic sidechains provide a template mediating PKC-peptide recognition; the other interactions listed in Table IV are specific to human PKCa and a few isoforms of PKC.

Orr et al.\(^{72}\) have shown that PKC\(\beta\)II is cleaved at Arg 19 and that the pseudo-substrate is exposed upon in vitro activation by protamine sulphate. In the human PKCa model, Arg 19 is inaccessible and is surrounded by acidic sidechains Asp 539, Glu 541, Asp 542, Glu 543, Asp 544, and Glu 545, as also noted by Orr and Newton.\(^{71}\) Hence these observations made for PKC\(\beta\)II are likely to be valid also for PKCa.

The residues in cPKI that interact with or in the vicinity of MnATP are Thr 51, Lys 72, Glu 91, Glu 127, Lys 168, and Asp 184 (PKC numbering: 347, 368, 387, 424, 465, and 481, respectively). All except Thr 51, which is in the Gly-rich loop, and Glu 127 are conserved in human PKCs. These residues are replaced by Lys and Asp, respectively. These changes are not likely to affect the MnATP binding in PKCa.

### Calcium-Dependent Lipid-Interacting Domain

The calcium-dependent lipid-interacting domain (C2 region) occurs in the isoforms of PKCs that are calcium dependent, thus implying that the C2 region is a potential calcium-regulatory domain. The C2 region occurs in over 40 other proteins, notably synaptotagmin, PKC-related kinases (PRKs), phospholipase C, phospholipase A2, rabphilin, and GTPase-activating proteins, suggesting that the C2 domain is a common calcium-regulatory domain.\(^{24,73-78}\) Sequence identities between the C2 domains of members of the synaptotagmin and PKC families range from 24 to 38%. However, the amino acid sequence of the C2 domain shows no similarity to other calcium-binding motifs such as E-F hands.

The crystal structure of the N-terminal C2 domain of rat synaptotagmin I was recently reported at 1.9 Å resolution.\(^{25}\) Synaptotagmin has two C2

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**TABLE IIIA.** Residues in PKC That Are Involved in the Recognition of a Pseudosubstrate (equivalent residues in CAPK are also indicated)

<table>
<thead>
<tr>
<th>Residue no.</th>
<th>PKC-α</th>
<th>CAPK</th>
<th>Diff. in volume (Å(^3))*</th>
</tr>
</thead>
<tbody>
<tr>
<td>349</td>
<td>Ser</td>
<td>Ser</td>
<td>0</td>
</tr>
<tr>
<td>350</td>
<td>Phe</td>
<td>Phe</td>
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<tr>
<td>379</td>
<td>Asp</td>
<td>Lys</td>
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<tr>
<td>380</td>
<td>Asp</td>
<td>Glu</td>
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<tr>
<td>424</td>
<td>Asp</td>
<td>Glu</td>
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<td>426</td>
<td>Met</td>
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<td>430</td>
<td>Gln</td>
<td>Arg</td>
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<td>465</td>
<td>Lys</td>
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</tr>
<tr>
<td>467</td>
<td>Asp</td>
<td>Glu</td>
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<tr>
<td>350</td>
<td>Pro</td>
<td>Pro</td>
<td>0</td>
</tr>
<tr>
<td>350</td>
<td>Ile</td>
<td>Leu</td>
<td>0</td>
</tr>
<tr>
<td>530</td>
<td>Glu</td>
<td>Glu</td>
<td>0</td>
</tr>
<tr>
<td>543</td>
<td>Glu</td>
<td>Pro</td>
<td>+19</td>
</tr>
<tr>
<td>547</td>
<td>Phe</td>
<td>Tyr</td>
<td>-6</td>
</tr>
</tbody>
</table>

*Sidechain volume of a residue in CAPK is subtracted from the equivalent residue in the pseudosubstrate of PKC-α. The positive or negative sign is attached accordingly.

**TABLE IIIB.** Sidechains in PKC Pseudosubstrate That Are Involved in the Recognition by the Enzyme (equivalent residues in PKI peptide recognized by CAPK are also indicated)

<table>
<thead>
<tr>
<th>Residue no.</th>
<th>PKC-α pseudosubstrate</th>
<th>CAPK-PKI peptide</th>
<th>Diff. in volume (Å(^3))*</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Phe</td>
<td>Thr</td>
<td>+42</td>
</tr>
<tr>
<td>22</td>
<td>Arg</td>
<td>Arg</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>Lys</td>
<td>Arg</td>
<td>-13</td>
</tr>
<tr>
<td>25</td>
<td>Ala</td>
<td>Ala</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>Leu</td>
<td>Ile</td>
<td>0</td>
</tr>
<tr>
<td>27</td>
<td>Arg</td>
<td>His</td>
<td>+30</td>
</tr>
</tbody>
</table>

*Sidechain volume of a residue in the PKI peptide is subtracted from the equivalent residue in the pseudosubstrate of PKC-α. The positive or negative sign is attached accordingly.
domains the fold of which are eight-stranded Greek-key β-sandwiches with a novel connectivity pattern of β-strands. Connection between strands 5 and 6 accommodates the only helical segment in the structure (other than a Gly-rich helix tagged before the N-terminus of the C2 domain). The calcium-binding ligands come from two loops separated in the amino acid sequence, but proximal in the tertiary structure. In the N-terminal C2 domain they include sidechains of Asp 230, Asp 232 (partially ordered in the structure) and Asp 178, the backbone carbonyl at Phe 231, and a water molecule. Both the carboxylates of Asp 230 can coordinate the calcium ion. The coordination sphere also includes a partially ordered Asp 172. The crystal structure of the calcium-free form is identical to the calcium-bound form except for the different orientation of the sidechain of Asp 232.25

Figure 7A shows the alignment of the sequence of the C2 domain region of human PKCa with the N- and C-terminal C2 domains of synaptotagmin. The structural environments at each residue of the N-terminal C2 domain of synaptotagmin are also shown. The sequence identity between PKCa and the N-terminal C2 domain of synaptotagmin is 34%, and that between the two C2 domains of synaptotagmin is 38%. We scored the equivalence of residues in the non-identical residue positions using a 20 × 20 mutation matrix. Considering such substitution probability of the amino acids, the sequence similarity between the C2 domain of PKCa and synaptotagmin is 69%, implying that in most places the sidechains are conservatively replaced. Insertions and deletions in the alignment between the N-terminal C2 domain of synaptotagmin and PKCa occur in loop regions. A three-dimensional model for the C2 domain of PKCa has been generated and energy minimized (Fig. 7B). All the regular secondary structure regions and the calcium-binding residues are well accommodated in structurally conserved regions, although the calcium-binding residues occur in two solvent-exposed loops. The four structurally variable regions of PKCa (compared with synaptotagmin) are 170–171, 204–208, 237–238, and 267–268, and these have been modeled on 2GD1 P
TABLE IV. List of Possible Interactions Between PKC and a Pseudosubstrate

<table>
<thead>
<tr>
<th>PKC-α</th>
<th>Pseudosubstrate</th>
<th>Nature of interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>O Lys 347</td>
<td>NH1 Arg 22</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>OG Ser 349</td>
<td>O Ala 25</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>CD2 Phe 350</td>
<td>CB Ala 25</td>
<td>Hydrophobic interactions</td>
</tr>
<tr>
<td>O Asp 378</td>
<td>NE Arg 27</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>OD2 Asp 379</td>
<td>O Lys 29</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>OD1 Asp 380</td>
<td>NH2 Arg 27</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>OD1 Asp 424</td>
<td>NE Arg 22</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>SD Met 426</td>
<td>CB Phe 20</td>
<td>Sulfur-aromatic interaction</td>
</tr>
<tr>
<td>OE1 Gln 430</td>
<td>O Phe 20</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>NZ Lys 465</td>
<td>O Lys 23</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>OD1 Asp 467</td>
<td>NE Arg 22</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>OD2 Asp 467</td>
<td>NH1 Arg 22</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>CE Met 484</td>
<td>CB Ala 25</td>
<td>Hydrophobic interaction</td>
</tr>
<tr>
<td>OE3 Thr 497*</td>
<td>N2 Lys 29</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>CD2 Phe 498</td>
<td>CD2 Leu 26</td>
<td>Hydrophobic interaction</td>
</tr>
<tr>
<td>O Gly 500</td>
<td>N Leu 26</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>CD Pro 502</td>
<td>CD2 Leu 26</td>
<td>Hydrophobic interaction</td>
</tr>
<tr>
<td>CD1 Ile 505</td>
<td>CD2 Leu 26</td>
<td>Hydrophobic interaction</td>
</tr>
<tr>
<td>OE2 Glu 530</td>
<td>NZ Lys 23</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>OE1 Glu 543</td>
<td>NH2 Arg 19</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>CZ Phe 547</td>
<td>CD2 Leu 26</td>
<td>Hydrophobic interaction</td>
</tr>
</tbody>
</table>

*Thr 497 is phosphorylated, and OE3 corresponds to one of the oxygens in the phosphate group.

67–68, 1PAZ 82–86, 2BJL 1 7–8, and 1FX1 42–43, respectively.

Replacements of exposed hyd...dies in synaptotagmin (e.g., Ile 159, Ile 163, Phe 184, and Phe 193) are by more suitable polar residues in PKCa (Fig. 7A). From the model, it appears that these replacements are also accompanied by slightly different tertiary interactions stabilizing the fold of the C2 domain. For example, in synaptotagmin there are β-bulges at positions Ile 163-Gln 164 and Val 205-Phe 206-Asn 207 occurring in two successive β-strands, which are in register, forming part of a β-sheet. Ile 163 and Val 205 that are exposed in synaptotagmin are replaced by Arg and Gln, respectively, in PKCa. These changes could modify the β-bulge conformation. However, another β-bulge in synaptotagmin at Gly 241 that introduces a sharp bend in a β-ribbon to form one of the calcium-binding loops is probably conserved in PKCa. In synaptotagmin Thr 195 is buried (Fig. 7A) and introduces a severe distortion of β-structure at the edge of the sheet. This Thr is conserved in PKCa (Fig. 7A) and also in all known protein sequences with C2 domain.

The calcium-binding sidechains or those within the coordination sphere (Asp 172, 178, 230, and 232) of synaptotagmin are absolutely conserved in PKCa (Fig. 7A). Hence the calcium-binding mode of PKCa is expected to be identical to that of synaptotagmin. We have used the calcium-free form of synaptotagmin crystal structure, which was kindly provided by Dr. Stephen Sprang, to model the C2 domain of PKCa. The major difference between the calcium-bound and -unbound crystal structures of synaptotagmin is the position of the sidechain Asp 232. The equivalent sidechain in PKCa, Asp 248 (232), has been remodeled to bring the carboxylates into the coordination sphere. Figure 7B shows a representation of the C2 domain of PKCa with the modeled calcium and liganded sidechains.

In the crystal structure of synaptotagmin the region between 189 and 192 involving four consecutive lysine residues is not well defined in the electron density and disordered. Since the region is abundant in positive charge, Sutton et al. suggest that it could involve phospholipid interactions. The C2 domain of PKCa also interacts with phospholipid. Although there is no stretch of poly-lysine, the amino acid sequence at the equivalent loop of PKCa, Lys-Asn-Glu-Ser-Lys-Gln-Lys-Thr-Lys, shows a preponderance of basic residues. Figure 7B highlights this region in our model; it can be seen that it is largely exposed.

Zinc- and Activator-Binding Modules

Both the activators diacylglycerol and phorbol ester bind at the two zinc-binding domains of PKCa. The NMR structure of the second of the zinc-binding domains in rat PKCδ (uncomplexed with activators) consists of two antiparallel β-sheets and a short helical segment that packs against one of the sheets. The two metal centers are present at either end of this sheet, coordinated by Cys and His residues. The NMR structure did not define the positions of the
Fig. 7. A: Alignment of amino acid residues 156–292 of human PKCa (PKC-A), residues 139–269 of rat synaptotagmin I (SYT-N), and residues 270–408 of rat synaptotagmin I (SYT-C). Structural environments of residues as in the crystal structure of synaptotagmin (SYT-N) are indicated using the program JOY. The residue numbering at top corresponds to that of Sutton et al. The residue numbering at bottom is that of PKCa. Explanation for JOY notation is given in Figure 2. B: Model of the C2 domain of PKCa. β-strands are shown as green arrows and sidechains of aspartates in the vicinity of calcium (yellow) are shown in red. Potential region for interaction with phospholipid is highlighted. The figure has been prepared using SETOR.
N-terminal segment (about seven residues) in the construct employed; this has an overlap with the pseudo-substrate region. The position of a short C-terminal segment was also undefined. However, the structurally defined module (residues 13–62, in the numbering followed by Hommel et al.20) includes all structural determinants for the binding of two zinc ions as well as phorbol ester.81

The crystal structure of the second of the zinc-binding modules in PKCβ has also been reported at 1.95 Å resolution.21 The length of this construct is shorter than that of Hommel et al.20 by three residues at the N-terminus and by one residue at the C-terminus, and the sequence identity between the two is 62%. The essential determinants of zinc and phorbol ester/diacyl glycerol binding are also present in the crystal structure construct. Indeed, crystal structures of both the complexed form with phorbol ester (at 2.2 Å resolution) and the uncomplexed form are reported.21 The complexed and uncomplexed crystal structures are almost identical as regions 21–24 and 33–36 (Hommel et al.20 numbering) where the NMR structure differs significantly from the crystal structure. The crystal structure differs significantly from NMR structure primarily in two regions; one is the “pulling apart” of the segments in and around the second and third β-strands in the crystal structure, and the other is at the C-terminus. Both these regions are not well defined in the NMR structure. In the crystal structure some of the hydrophobic residues (Tyr 20, Met 21, Phe 25, and Trp 34—Hommel et al.20 numbering) in the former region are well ordered as they are involved in contact with adjacent molecules in the crystal lattice.21 Phorbol ester binds in the gap formed between the regions 21–24 and 33–36 (Hommel et al.20 numbering) where the NMR structure differs significantly from the crystal structure.

The amino acid sequence of the zinc-binding domain from rat PKCβ studied by NMR is identical to the corresponding region in human PKCa (residues 99–152; Fig. 1). The tertiary structure for the first zinc-binding domain in the sequence of human PKCa was modeled initially on the basis of the NMR structure but later on the basis of the crystal structure (see Materials and Methods for details). Figure 8 shows the alignment between the three sequences with the structural features in the NMR and crystal structures represented. The sequence of the modeled module is highly similar to those of NMR (38%) and X-ray (42%) structures with no insertions or deletions in the alignment except for the C-terminal glycyl residue in the NMR structure, which has a positive φ conformation tending to align with a prolyl residue in the model.

The Cys and His ligands that bind to the two zinc ions are conserved within the three sequences (labeled as “Z” in Fig. 8). In the crystal structure (PKCβ) Tyr 238 (20), Leu 251 (33), and Gln 257 (39) form the bottom of the phorbol ester binding groove. The sides of the groove are formed by mainchain atoms from 259–242 (21–24) and 250–254 (32–36) and the sidechain atoms of Pro 241 (23), Thr 242 (24), Leu 250 (32), and Leu 254 (36). The polar interactions between PKC and phorbol ester are essentially hydrogen bonds involving mainchain carbonyls and amides. As in the crystal structure, both the amide and carbonyl of Gly 59 (35) in the model (ZN1-MDL in Fig. 8), which adopts φ,ψ values disallowed for non-glycyl residues, is hydrogen bonded to two of the oxygens in the five-membered ring of phorbol ester. Occurrence of Gly at this position is crucial for the recognition of phorbol ester and is also conserved in the second zinc-binding module of PKCβ. The backbone amide and carbonyl at Thr 113 (24) in the second zinc-binding domain are hydrogen bonded to the hydroxyl at a carbon in the seven-membered ring of the ligand, and these interactions observed in the crystal structure are conserved in the model. As the amino acid sequences of the two zinc-binding modules of PKCa are highly similar, this interaction is also expected in the first zinc-binding domain. This hydroxyl is also hydrogen bonded to the carbonyl at position 122 (33). The importance of mainchain conformation in the recognition of phorbol ester is emphasized by Pro 241 (23) of the crystal structure. The homologous domains in non-phorbol ester-binding Vav and PKC’s that are not activated by phorbol ester have this Pro replaced. This substitution relieves rigidity in the backbone conformation, disrupting the hydrogen bonds with phorbol ester. In the crystal structure, sidechains of Pro 241 (23), Leu 250 (32), and Trp 252 (34) are involved in hydrophobic contact with the five-membered ring of phorbol ester, and all these residues are conserved in the second zinc-binding domain of PKCa except Trp 252, which is replaced by Tyr. In the first zinc-binding domain Leu 250 is replaced by Phe 56, and these changes are not likely to affect the interaction. In the crystal structure Tyr 238 (20) and a part of Leu 251 (33) interact with the seven-membered ring of phorbol ester. While these residues are conserved in the second zinc-binding domain of PKCa, they are replaced by easily substitutable Tyr and Ile in the first zinc-binding domain.

Phorbol ester and diacylglycerol have almost equal affinity for PKC, and they were thought to bind competitively through sterically equivalent interactions.82 The hydroxyl in the seven-membered group of phorbol ester that is involved in both donating and accepting hydrogen bonds is equivalent to 3-hydroxyl of diacylglycerol.21

Spatial Arrangement of Functional Modules—A Hypothetical Model

Several possible spatial arrangements of these two zinc-binding domains have been investigated. Packing involving side-by-side orientation of secondary structures cannot be completely ruled out.
Fig. 8. Alignment of the amino acid sequence of the second of the zinc-binding domains of PKCδ and PKCe with that of the first zinc-binding domain (ZN1-MDL) in PKCa. The structural environments of the residues as in the crystal structure of the second zinc-binding domain of PKCE12' (ZN2-CRY) and as in the NMR structure of PKCpZ0 (ZN2-NMR) are represented using the program JOY. Key to JOY notation is given in Figure 2. Z represents the shortness of the connecting peptides between the first and second zinc-binding domains and the C2 domain suggests that these three structural domains may be linked together as a single rigid structural entity (Fig. 1). The connecting peptide between the second zinc-binding domain and the C2 domain consists of only four residues (153–156), suggesting that these domains are spatially proximal. We modeled the interaction between the second zinc-binding domain and the C2 domain using two constraints: 1) the C-terminal of the second zinc-binding domain and the N-terminal of the C2 domain should be close enough to be linked by a four-residue segment; and 2) the calcium/phospholipid-binding region of C2 domain should point in the same direction as the activator binding region of the two zinc-binding domains. Two small hydrophobic patches located on the domains (Phe 104, Ile 106, Met 130, Val 144, and Ile 145 in the zinc-binding domains and Ile 184, Pro 185, Met 186, Gly 190, Leu 191, and Leu 219 on the C2 domain) are buried in the interaction between the domains.

However, antiparallel packing is not possible, as the distance between the carboxy terminus of the first module and the amino terminus of the second module cannot be bridged by the 11 residues connecting the two modules. Two symmetrical possibilities were particularly attractive; two modules were related by a pseudo-two-fold such that the packing was between (1) helical segments and (2) extended structures. Both allowed access to the phorbol ester-binding region. However, helix-helix packing involved unfavorable sidechain-sidechain contacts such as Ile-Glu and Asn-Phe in the interface. On the other hand, packing of the extended structures gave favorable contacts between aromatic sidechains and between polar groups (Fig. 9).

The shortness of the connecting peptides between the first and second zinc-binding domains and the C2 domain suggests that these three structural domains may be linked together as a single rigid structural entity (Fig. 1). The connecting peptide between the second zinc-binding domain and the C2 domain consists of only four residues (153–156), suggesting that these domains are spatially proximal. We modeled the interaction between the second zinc-binding domain and the C2 domain using two constraints: 1) the C-terminal of the second zinc-binding domain and the N-terminal of the C2 domain should be close enough to be linked by a four-residue segment; and 2) the calcium/phospholipid-binding region of C2 domain should point in the same direction as the activator binding region of the two zinc-binding domains. Two small hydrophobic patches located on the domains (Phe 104, Ile 106, Met 130, Val 144, and Ile 145 in the zinc-binding domains and Ile 184, Pro 185, Met 186, Gly 190, Leu 191, and Leu 219 on the C2 domain) are buried in the interaction between the domains.

Packing between the dimer of zinc-binding modules and the catalytic region is severely constrained by the presence of only four residues connecting the last residue of the pseudo-substrate and the first residue in the first of the zinc-binding modules. Analysis of the surface properties suggested a potential spatial arrangement represented in Figure 10. Many other possibilities with the distance constraint between the pseudo-substrate and the first of the zinc-binding modules resulted in severe short contacts between the catalytic region and zinc-bind-
Fig. 9. A hypothetical model for the spatial arrangement of the two zinc-binding modules in the regulatory region. The two zinc-binding modules are distinguished by different colors of the backbone and sidechains in the two copies. Some of the residues in the interface are also shown. Figure prepared using SETOR.8

ing domains. This packing arrangement allows access to the activator binding region of zinc-binding domains as well as the phosphorylation site in the catalytic region.

In the present model the three putative lipid-binding sites in three of the domains are pointing in the same direction. The autoinhibitory pseudo-substrate is proximal to the N-terminal of the first zinc-binding domain. The positively charged amino acids flanking Ala 21 in the pseudo-substrate might interact with phospholipid head groups when PKC is activated. The length of the connection between the C2 domain and catalytic region and the fact that the regulatory region can be cleaved from the catalytic region suggests that apart from the pseudo-substrate there are no very strong interactions between the three regulatory domains and the catalytic region. The overall shape of the model is largely consistent with the electron microscopy studies.83

CONCLUSIONS

The analysis of the structure of protein kinase Ca provides a rational basis for designing experiments. For example, the identification of the template for enzyme-peptide recognition, probably common to most PKCs, can be tested. Several specific predictions of the analysis, i.e., the phosphorylation site in the catalytic region and the binding site, are entirely consistent with the mutagenesis experiments.16,17 This three-dimensional model also explains many experimental observations, as seen in the case of Ala 25 → Glu mutant in the pseudosubstrate. Predicted variations in the three-dimensional structure provide clues about a suitable probe structure for molecular replacement in the crystal structure determination of PKC isoforms.

The proposed docking of the two zinc-binding domains from the regulatory region suggests that activator binding may occur in the two inter-loop grooves that are exposed in the symmetric arrangement. This would have significant impact on our understanding of this pharmacophore. The model of the spatial arrangement of the various domains and the suggested exposed and proximal nature of binding sites provide a starting model that can be refined through experimentation.

The structure of a protein that interacts with PKC, 14-3-3, has recently been determined.84,85 While the role of 14-3-3 in regulating PKC activity in vivo is still not precisely defined, the recent demonstration that 14-3-3 can form heterodimers86 suggests that a 14-3-3 heterodimer might serve as a scaffold, bringing PKC into contact with a substrate such as Raf.87 The insights afforded into the PKCa protein will both drive and provide a rational basis for understanding the critical kinase-substrate and
Fig. 10. Schematic of the hypothetical model for the spatial arrangement of catalytic region and the regulatory region. Each of the functional units is highlighted in a given colour; the pseudo-substrate is in yellow, and the amino and carboxy termini of each of the modules are presented by N and C followed by a number. The continuity of the number represents connectivity of these modules in the primary structure of PKCa. Figure prepared using SETOR.90

perhaps kinase-binding protein interactions that underlie its biological function.

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