A Single Residue in the C1 Domain Sensitizes Novel Protein Kinase C Isoforms to Cellular Diacylglycerol Production

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The C1 domain mediates the diacylglycerol (DAG)-dependent translocation of conventional and novel protein kinase C (PKC) isoforms. In novel PKC isoforms (nPKCs), this domain binds membranes with sufficiently high affinity to recruit nPKCs to membranes in the absence of any other targeting mechanism. In conventional PKC (cPKC) isoforms, however, the affinity of the C1 domain for DAG is two orders of magnitude lower, necessitating the coordinated binding of the C1 domain and a Ca2+-regulated C2 domain for translocation and activation. Here we identify a single residue that tunes the affinity of the C1 domain for DAG (but not phorbol ester)-containing membranes. This residue is invariant as Tyr in the C1b domain of cPKCs and invariant as Trp in all other PKC C1 domains. Binding studies using model membranes, as well as live cell imaging studies of yellow fluorescent protein-tagged C1 domains, reveal that Trp versus Tyr toggles the C1 domain between a species with sufficiently high affinity to respond to agonist-produced DAG to one that is unable to respond to physiological levels of DAG. In addition, we show that while Tyr at this switch position causes cytosolic localization of the C1 domain under unstimulated conditions, Trp targets these domains to the Golgi, likely due to basal levels of DAG at this region. Thus, Trp versus Tyr at this key position in the C1 domain controls both the membrane affinity and localization of PKC. The finding that a single residue controls the affinity of the C1 domain for DAG-containing membranes provides a molecular explanation for why 1) DAG alone is sufficient to activate nPKCs but not cPKCs and 2) nPKCs target to the Golgi.

Protein kinase C (PKC) is a critical transducer of intracellular signaling pathways, with a variety of outputs, most notably tumor promotion (1, 2). The hallmark of PKC activation is its translocation to membranes (3). This translocation is mediated through the ligand-dependent engagement of two membrane-targeting modules: the C1 (ligand: diacylglycerol or DAG) and C2 (ligand: Ca2+) domains. The regulatory domains vary within the PKC family, which is subdivided into three groups based on regulation. The conventional PKC isoforms (cPKCs: α, βI, II, γ) contain two tandem C1 domains and one conventional C2 domain; consequently, cPKCs are regulated both by DAG and Ca2+. The novel isoforms (nPKCs: δ, ε, η, and θ) contain two tandem C1 domains and a non-Ca2+/membrane-binding novel C2 domain; consequently, nPKCs are regulated only by DAG. Atypical PKCs (aPKCs: ε and η) contain a single non-DAG-binding (“atypical”) C1 domain and no C2 domain; as a result, aPKCs are regulated by neither DAG nor Ca2+ (4).

The C1 domain is an ~8-kDa domain that binds DAG or the potent DAG-mimicking phorbol esters, such as phorbol-12-myristate-13-acetate (PMA) and phorbol dibutyrate (PDBu) (5). Structural studies have established that all C1 domains have a similar fold (6–11). An “unzipped” β sheet forms a groove lined by hydrophobic residues in which membrane-embedded diacylglycerol or phorbol esters bind (7). Membrane interaction is also facilitated by a ring of positive charges around the middle of the domain that potentially interacts with phosphatidylserine (PS) and other anionic lipids (12).

Nearly all C1 domains have been shown to bind PS (13), but their affinity for ligand (DAG/phorbol esters) varies substantially (14). The C1 domain of nPKCs has an intrinsic affinity for DAG-containing membranes 2 orders of magnitude higher than that of the C1 domain of cPKCs, allowing nPKCs to respond to agonists that trigger diacylglycerol production alone (15). In contrast, cPKCs must be pretargeted to membranes by their C2 domain in response to an elevation of intracellular Ca2+ to respond to DAG (16). The molecular basis by which nPKC C1 domains bind to DAG membranes with two orders of magnitude higher affinity than those of cPKC C1 domains has not been elucidated.

In this report, we identify a single conserved residue at position 22 in the C1 domain that tunes its affinity for DAG-containing membranes. Our findings provide a molecular basis for why nPKCs respond to DAG alone, whereas cPKCs require the coordinated elevation of Ca2+.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol, and 1,2-sn-dioleoyl-glycerol in chloroform were from Avanti Polar Lipids, Inc. Tritiated 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine was from American Radiochemical Co. [γ-32P]ATP was from PerkinElmer Life Sciences. PMA, PDBu, and BAPTA/AM were from CalBiochem. Glutathione-Sepharose 4B and PreScission Protease were from Amersham Biosciences. Electrophoresis reagents were from Bio-Rad. Oligonucleotides were from GenBase, Inc. Restriction
enzymes were from New England Biolabs, Inc. All other reagents and chemicals were reagent-grade.

Sequence Alignments—Sequences of the C1 domains of PKC isoforms (13) were aligned using CLUSTALW from the LaserGene 6 software package (DNASTAR, Inc.). Residues that contact ligand in the structure of C1bδ with phospholipid were taken from the LIGPLOT file provided through the Protein Data Bank (PDB) repository (PDB ID: 1PTR).

Construction of Plasmids and Protein Purification—For bacterial expression, C1bβ was cloned into pGEX-KG as described (17). C1bδ (Gln-221 to Ala-290) and C1bβ were subcloned into pGEX-6P3, in which the Tyr-22  arrow Trp mutation was introduced. For mammalian expression, all constructs were cloned into pcDNA3. PM-CFP was cloned as described (18). YFP was fused to the 3' end of C1bβ (Pro-93 to Gly-152) and C1bβ (Phe-225 to Gly-281) to make C1bβ-YFP and C1bδ-YFP. These constructs were used to produce the mutants Tyr22Trp (C1bβ) and Trp-22  arrow Tyr (C1bδ). YFP was fused to the 3' end of PKCβII or δ, and Tyr123Trp or Trp252Trp mutations were introduced, respectively. Rat PKCβII and murine PKCδ isoforms were used in these studies. All mutations were made using QuikChange (Stratagene). Wild-type C1bδ was expressed in bacteria and purified as described previously (17). The C1bβII-Y123W and C1bδ domains were purified similarly (17), with the substitution of PreScission Protease for thrombin.

Sucrose-loaded Vesicle Binding Assay—Lipid vesicles were prepared and PMA were incorporated into these vesicles as described (15). The final concentration of lipid was determined by phosphate analysis as described (19). The binding of the C1b domain to sucrose-loaded large unilamellar vesicles was measured as described (20). To normalize data between 0 and 100% bound, curves were fitted to Equation 1,

\[
\text{Fraction bound} = \frac{(n[L]^{i/n}) + K_d}{(n[L]^{i/n} + K_d)} + \text{int}
\]

where \(K_d\) is the apparent equilibrium constant, \(L\) is the lipid concentration, \(H\) is the Hill coefficient, \(n\) is the range of apparent percent bound, and \(\text{int}\) is the \(y\) intercept. Experiments were performed in triplicate. Measurements at each lipid concentration were averaged, and data were fitted to one master S.E.-weighted plot of the following form,

\[
\text{Fraction bound} = \frac{(L)^{i/n}(L)^{i/n} + K_d^{i/n}}{(L)^{i/n} + K_d^{i/n}}
\]

where the terms represent the same parameters as in Equation 1. All curve fitting was done using Kaleidagraph v5.32.

Cell Culture—COS7 cells were plated in Dulbecco's modified Eagle's medium (Cellgro) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO\(_2\). Cells were plated in 35-mm imaging dishes at 60% confluence and transfected using FuGENE 6 (Roche Diagnostics). Cells were cotransfected with PM-CFP and C1b-YFP constructs for translocation studies. For localization experiments, the YFP-tagged constructs were transfected alone. Cells were allowed to grow for 12–24 h post-transfection before imaging. For kinase activity assays, cells were transfected with full-length YFP-tagged constructs (to monitor expression) and allowed to grow for 48 h post-transfection before harvesting.

Cell Imaging—Cells were imaged as described previously (18, 21). Because of cell-to-cell variability in CFP and YFP expression, the dynamic range varied from cell to cell. However, treatment with phospholip esters caused maximal membrane binding of each domain (Fig. 1C). Therefore, the responses to DAG generation via UTP were calibrated to the dynamic range of the cell by dividing each point by the maximal response elicited by PDBu. Thus, the data are presented in relative translocation units.

Kinase Assay—Lysates from untransfected COS7 cells or COS7 cells transfected with PKCβII, PKCβII-Y123W, or PKCδ were assayed for PKC activity by monitoring phosphorylation of a synthetic peptide as described (22). Non-activating conditions contained 20 mm HEPES (pH 7.5) and 2 mm EGTA. Activating conditions contained 140 μM PS, 3.8 μM DAG, and either 2 mm CaCl\(_2\) (total PKC activity) or 2 mm EGTA (Ca\(^{2+}\)-independent PKC activity).

Molecular Modeling—The structure of the C1 domain was visualized and manipulated using Swiss-PdbViewer, v3.7. Coordinates for C1-Raf (1FAR), C1b-PKCγ (1TBN), and C1b-PKCδ (1PTQ) were taken from the PDB repository (PDB ID numbers in parentheses). The C1 domain of human PKCζ was modeled on the C1 domains of DGKδ (1R79), Ksr (1KBE), β2-chimaerin (1XA6), munc-13 (1Y8F), PKCδ, PKCγ, and Raf, using Swiss-PdbViewer (23–25).

RESULTS AND DISCUSSION

The C1 domains of nPKCs bind DAG-containing membranes with 2 orders of magnitude higher affinity than those of cPKCs (15). Alignment of the sequences of C1 domains of cPKCs and nPKCs revealed that the residue at position 22 is invariant as Trp in all C1 domains except the C1b domains of cPKCs, where it is invariant as Tyr (Fig. 1A). This residue lies along one of the two loops that bind ligand and is on a surface that interacts with the membrane (12). Thus this is a candidate to modulate ligand-dependent membrane affinity.

To test the hypothesis that position 22 controls the affinity of the C1b domain for lipid membranes, we first mutated Tyr-22 to Trp in the C1b domain of the cPKC β/II (C1bβ-Y22W) and measured the binding of the bacterially purified wild-type and mutant domains to lipid vesicles containing 30 mol % PS and 5 mol % DAG (Fig. 1B, filled symbols). Wild-type C1bβ (C1bβ-WT) bound to vesicles containing 5 mol % DAG with a \(K_d\) of 780 ± 50 μM (filled diamonds). On the other hand, the mutant C1bβ-Y22W bound with 31-fold higher affinity to vesicles of the same composition (\(K_d\) = 24 ± 1 μM, filled squares). Binding was dependent on the presence of DAG, as the domain did not bind to 500 μM lipid vesicles containing 30 mol % PS and 0 mol % DAG (data not shown). We next measured the binding of these two domains to vesicles containing 30 mol% PS and 1 mol % PMA (Fig. 1B, open symbols). C1bδ-WT and -Y22W bound with the same affinity to vesicles containing 1 mol % PMA (\(K_d\) values of 35 ± 3 and 35 ± 2 μM, open diamonds and squares, respectively). These data reveal that mutation of Tyr-22 to Trp in the C1 domain of PKCβ converts the domain from a low affinity to a high affinity DAG-binding module.

We next tested whether the isolated C1b domain of the nPKC δ (C1bδ), which has a Trp at position 22, also has higher intrinsic affinity for DAG-containing membranes than C1bβ. We used a GST-tagged construct for C1bδ, as removal of the
GST tag greatly reduced the stability of the protein. GST-C1bδ bound to vesicles containing 5 mol% DAG and 1 mol% PMA with $K_d$ values of $35 \pm 3$ and $56 \pm 6 \mu M$, respectively (Fig. 1B, triangles). Low, but pure, amounts of untagged C1bδ showed that the GST tag had no effect on the affinity of the C1bδ for DAG- or PMA-containing membranes (data not shown). Moreover, while C1bβ-WT showed a 110-fold preference for PMA over DAG on an equimolar basis (Fig. 1B, open versus closed diamonds), C1bβ-Y22W and GST-C1bδ showed modest 3.6- and 3.1-fold selectivities for PMA over DAG, respectively (Fig. 1B, open versus closed squares for C1bβ-Y22W and triangles for GST-C1bδ).

We previously observed that the C1 domain exhibits selectivity among anionic lipids in the presence of DAG, with preference for PS over phosphatidylglycerol (PG) (17). Since residue 22 is important for sensitizing the C1b domain to DAG, this position should also allow the domain to discriminate between PS and PG. Therefore, we measured the affinity of the C1bβ-WT, C1bβ-Y22W and GST-C1bδ domains for membranes containing 30 mol% PS or PG and 5 mol% DG or PMA. Wild-type C1bδ showed 2-fold selectivity for PS over PG, regardless of whether the ligand was DAG or PMA (Table 1). Trp-containing C1 domains, however, showed high selectivity for PS, although only in the context of DAG membranes (5- and 20-fold selectivity for C1bβ-Y22W and GST-C1bδ, respectively).

TABLE 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_d$ ($\mu M$)</th>
<th>C1bβ</th>
<th>C1bβ-Y22W</th>
<th>C1bδ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DAG</td>
<td>PMA</td>
<td>DAG</td>
<td>PMA</td>
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<tr>
<td>PS</td>
<td>780 ± 50</td>
<td>11.5 ± 0.5</td>
<td>24 ± 1</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>PG</td>
<td>1690 ± 60</td>
<td>22 ± 1</td>
<td>130 ± 10</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>PG:PS</td>
<td>2.2 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>5.4 ± 0.5</td>
<td>1.1 ± 0.2</td>
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To monitor the real-time membrane translocation of isolated C1b domains in live cells, we fused YFP to the C terminus of C1bβ and C1bδ. We also generated YFP fusion constructs for these two C1 domains containing point mutations reversing the identity of residue 22: C1bβ-Y22W and C1bδ-W22Y. We cotransfected COS7 cells with CFP that had been targeted to the plasma membrane (PM-CFP) and the indicated YFP-tagged C1 domain construct; translocation to the plasma membrane was monitored as an increase in the ratio of FRET-based YFP emission: CFP emission (FRET ratio) (21).

UTP, acting through endogenous P2Y receptors, stimulates the production of DAG at the plasma membrane via phospholipase C-mediated lipid hydrolysis (26). Fig 1C shows that stimulation of COS7 cells with UTP (100 $\mu M$) resulted in an increase in FRET ratio, which was further increased to maximal translocation following addition of PDBu (200 nm). Upon stimulation of COS7 cells with UTP, C1bδ did not significantly translocate to membranes (Fig 1C, blue diamonds). In contrast, UTP stimulation caused robust translocation of C1bδ (Fig 1C, green triangles). Consistent with in vitro binding data (Fig. 1B), C1bβ-Y22W responded to UTP (Fig. 1C, red squares), resulting in a 10-fold increase in DAG binding at the plasma membrane relative to C1bβ-WT (Fig. 1C, blue diamonds). Conversely, mutating Trp-22 to Tyr in C1bδ (C1bδ-W22Y) reduced the translocation in response to UTP 10-fold relative to C1bδ without altering the maximal translocation driven by PDBu (Fig. 1C, yellow circles). These data reveal that Trp versus Tyr at position 22 in the C1b domain renders the domain responsive to DAG generated by receptor-mediated phospholipid hydrolysis.

To determine whether binding differences arising from changes in the C1b domain affected the cofactor dependence of the full-length kinase, we generated a full-length PKCβII construct in which Tyr-123 (position 22 of the C1b domain) was mutated to Trp (PKCβII-Y123W). We transfected COS7 cells with either this mutant construct, wild-type PKCβII, or PKCδ...
and assayed kinase activity from the detergent-soluble lysates compared with untransfected control cells. We assayed PKC activity in the presence of PS, DAG, and either Ca\(^{2+}\) (for total PKC activity) or EGTA (for Ca\(^{2+}\)-independent activity), and Ca\(^{2+}\)-independent activity was calculated as a percent of total PKC activity. Fig. 1D shows the Ca\(^{2+}\)-independent activity of PKC\(\beta\)II, PKC\(\beta\)II-Y123W, and PKC\(\delta\). Consistent with the standard model where Ca\(^{2+}\), DAG, and PS are required for full activation of cPKCs (27), PKC\(\beta\)II had minimal (6%) activity in the absence of Ca\(^{2+}\). In contrast, PKC\(\delta\) was activated to near maximal levels in the absence of Ca\(^{2+}\), consistent with the Ca\(^{2+}\)-independence of novel PKC isoforms (27). Strikingly, the single point mutation of Y123W in PKC\(\beta\)II was sufficient to confer significant Ca\(^{2+}\)-independent activity (28% of maximal activity). These results are consistent with the tighter membrane affinity conferred by Trp versus Tyr in the C1b domain, which results in reduced dependence on the C2 domain (and hence Ca\(^{2+}\)) for activation and is consistent with our previous studies, which showed that activation of PKC depends upon the affinity by which PKC binds to membranes (17). This provides a molecular explanation for why novel PKC isoforms are able to respond to DAG alone, while conventional PKC isoforms require pre-targeting by Ca\(^{2+}\) via their C2 domains for translocation and activation (15, 16).

Translocation of different PKC isoforms to discrete subcellular regions is an important mechanism for achieving specificity in PKC signaling. While the typical site of signaling for cPKCs is the plasma membrane (28), localization at endomembranes, particularly the Golgi, has been shown to be critical for PKC\(\delta\) activity (29). We observed striking differences in the localization of the isolated, YFP-tagged C1b\(\beta\) and C1b\(\delta\); C1b\(\beta\) was localized diffusely throughout the cell (Fig. 2A, upper left panel), while C1b\(\delta\) was concentrated at a juxtanuclear region resembling Golgi membranes (Fig. 2A, lower left panel). Localization at the Golgi was confirmed by treatment with brefeldin A, which abolished the juxtanuclear concentration of C1b\(\delta\) (data not shown). Moreover, the reversion mutants C1b\(\beta\)-Y22W and C1b\(\delta\)-W22Y showed a complete reversal of the subcellular localization of their wild-type counterparts (Fig. 2A, right panels). A previous study reported constitutive Golgi localization of the C1b domain of the nPKC\(\theta\) in unstimulated cells; this localization was redistributed to the cytosol upon inhibition of phospholipase C or phosphatidic acid phosphatase (30). Thus, the targeting effects of position 22 are likely due to DAG-dependent membrane binding.

Next, we tested whether having a Tyr or Trp at position 22 also affected the localization of either full-length PKC\(\beta\)II or PKC\(\delta\). PKC\(\delta\) prelocalized to juxtanuclear membranes, while PKC\(\beta\)II was basally cytosolic (Fig. 2B, left panels). Consistent with data from the isolated C1b domains, basal Golgi localization of the full-length PKC\(\delta\) was greatly diminished upon mutation of Trp-252 (position 22 of the C1b domain) to Tyr (Fig. 2B, lower right panel). However, mutation of Tyr-123 to Trp in PKC\(\beta\)II did not cause any change in its localization, as PKC\(\beta\)II remained cytosolic (Fig. 2B, upper panels). Thus, merely increasing the affinity of the C1b domain for DAG is not sufficient to determine the subcellular distribution of PKC. This suggests that other determinants control cPKC localization. For example, the Ca\(^{2+}\)-binding C2 domain of cPKCs, a feature absent from nPKCs, may override targeting to the Golgi (31, 32). Thus, in addition to affecting PKC function by regulating activation in response to DAG, position 22 of the C1b domain may also regulate DAG-dependent prelocalization of novel PKC isoforms. Taken together, these data suggest a model of activation in which low DAG levels allow prelocalization of PKC\(\delta\) to the Golgi through the C1b domain, while an additional agonist-stimulated increase in DAG shifts the equilibrium to full binding and full activation.

To gain insight into how Trp versus Tyr at position 22 controls the affinity of the C1 domain for DAG-containing membranes, we compared the backbone structures and molecular surfaces of several C1 domains (Fig. 3). We chose representatives of three C1 groups for modeling studies: those that bind DAG membranes with relatively high affinity (C1b\(\beta\)), those that bind DAG membranes with relatively low affinity (C1b\(\gamma\), a surrogate for C1b\(\beta\), with which C1b\(\gamma\) shares 80% identity and 92% similarity), and those that do not bind DAG (C1\(\theta\)). As shown in Fig. 3, comparison of the C1b domains of PKC\(\gamma\), -\(\delta\), and -\(\zeta\) reveals large movements within the \(\beta3/4\) loop (left panel). The \(\beta1/2\) and \(\beta3/4\) loops form the phorbol/DAG-binding pocket and contain the ligand- and membrane-binding determinants (7, 13). Residue 22 lies at the apex of this highly mobile \(\beta3/4\) loop, in keeping with its role as a critical regulator for the ability of the C1 domain to bind DAG.
Numerous elegant studies delineating the residues in the C1 domain involved in ligand binding have identified residue 22 as participating in DAG-dependent membrane binding (8, 14, 33, 34), yet the mechanism by which this position controls binding to membranes has not been clear. Our modeling studies suggest that position 22 may regulate the size of the ligand-binding pocket (Fig. 3). The phenol ring of Tyr-22 in C1bγ lies in a very different orientation relative to the membrane compared with the indole ring of Trp-22 in C1bδ. Moreover, these two amino acids are known to be positioned very differently at the water/lipid bilayer interface (35, 36), suggesting that mobility in the β3/4 loop may dictate the width and depth of the ligand-binding pocket. Indeed, several studies have shown flexibility within this loop, whereas the rest of the structure tends to remain static (7, 8, 37). The presence of two highly conserved Gly residues within the β3/4 loop (Gly-23 and Gly-28) suggests that such flexibility is not only possible but may also be required for function. Particularly relevant to our study, the ligand-binding cavity in C1bδ is narrow and deep, while that of the non-DAG responsive C1bγ is wide and shallow. Thus, whereas the smaller DAG can bind well to C1bδ, only the larger phorbol esters can make hydrophobic contacts across the wide gorge of C1bγ.

Curiously, the C1 domain of PKCζ has Trp at position 22, yet it still does not bind DAG. Rather, C1ζ appears to lose the ability to bind DAG by sterically and electrostatically occluding the ligand-binding pocket through substitution of hydrophobic residues (gray) for large basic amino acids (blue) (Fig. 3). Indeed, Blumberg and co-workers have shown that mutation of Asn-7, Ser-10, Pro-11, and Leu-20 in the β1/2 and β3/4 loops to arginine in C1bζ results in almost complete loss of binding to phorbol ester-containing membranes, while the reverse mutations in C1ζ confer phorbol ester responsiveness (38).

CONCLUSION

In this study, we identify Trp versus Tyr at residue 22 of the C1b domain as a molecular switch that controls whether PKC isoforms can respond to DAG alone or whether the coordinated binding of a second membrane-targeting module (i.e. the C2 domain of cPKCs) is required to confer responsiveness to agonist. Our findings provide a molecular basis for why nPKCs respond to DAG alone, whereas cPKCs require the coordinated elevation of Ca²⁺. This caution against drawing physiological conclusions when substituting phorbol esters for DAG in the context of cellular signaling, an idea that has also been suggested by other groups (33). Taken further, our results also suggest that therapeutic compounds designed to target the C1 domain of a specific isoform should more closely resemble DAG than phorbol, an approach taken by Blumberg and co-workers (39).

REFERENCES