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

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 Ca$$^{2+}$$-regulated C2 domain for translocation and activation. Here we identify a single residue that tunes the affinity of the C1b 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.

**Materials**—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-diacylglycerol), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine were from Avanti Polar Lipids, Inc. Tritiated -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 enzyme-mediated cloning was done at the King V. M. Davis Cancer Center DNA Sequencing Facility and fluorescent protein tagging was done at the University of California at San Diego. Materials, including reagents and equipment, were purchased from commercial vendors.

**EXPERIMENTAL PROCEDURES**

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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 C1 δ 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, C1 β was cloned into pGEX-KG as described (17). C1 δ (Gln-221 to Ala-290) and C1 β were subcloned into pGEX-6P3, in which the Tyr-22 → 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 C1 β (Pro-93 to Gly-152) and C1 δ (Phe-225 to Gly-281) to make C1 β-YFP and C1 δ-YFP. These constructs were used to produce the mutants Tyr22Trp (C1 β) and Trp22 → Tyr (C1 δ). YFP was fused to the 3′ end of PKC βII or δ, and Tyr123Trp or Trp252Tyr mutations were introduced, respectively. Rat PKC βII and murine PKC δ isoforms were used in these studies. All mutations were made using QuikChange (Stratagene). Wild-type C1 β was expressed in bacteria and purified as described previously (17). The C1 δ-III-Y123W and C1 δ domains were purified similarly (17), with the substitution of PreScission Protease for thrombin.

**Sucrose-loaded Vesicle Binding Assay**—Lipid vesicles were prepared and PMA was incorporated into these vesicles as described (15). The final concentration of lipid was determined by phosphate analysis as described (19). The binding of the C1 δ 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.

\[
\text{Fraction bound} = \frac{(n^*|L|)^n}{|L|^n + K_d^{int}} + \text{int} \tag{Eq. 1}
\]

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,

\[
\text{Fraction bound} = \frac{|L|^n}{|L|^n + K_d^m} \tag{Eq. 2}
\]

where the terms represent the same parameters as in Equation 1. All curve fitting was done using Kaleidograph 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% CO₂. 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 C1 δ-YFP constructs for localization 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 phorbol 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 PKC βII, PKC βII-Y22W, or PKC δ 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 CaCl₂ (total PKC activity) or 2 mM EGTA (Ca²⁺-independent PKC activity).

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

**RESULTS AND DISCUSSION**

The C1 δ 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 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 C1 δ domain for lipid membranes, we first mutated Tyr-22 to Trp in the C1 δ domain of the cPKC βII (C1 δ-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 C1 β (C1 β-WT) bound to vesicles containing 5 mol % DAG with a \(K_d\) of 780 ± 50 μM (filled diamonds). On the other hand, the mutant C1 β-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). C1 β-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 PKC β converts the domain from a low affinity to a high affinity DAG-binding module.

We next tested whether the isolated C1 δ domain of the nPKC δ (C1 δ), which has a Trp at position 22, also has higher intrinsic affinity for DAG-containing membranes than C1 δ. We used a GST-tagged construct for C1 δ, as removal of the
FIGURE 1. Residue 22 tunes binding of the C1b domain to DAG membranes in vitro and in vivo and affects kinase activity. A, sequence alignment of the C1 domains of cPKC and nPKC isoforms. Position 22 is boxed in yellow for Tyr and green for Thr. Residues that contact phorbol and/or form the hydrophobic wall of the groove in which DAG or phorbol binds are marked below with an “X.” B, binding of C1β-WT (blue diamonds) and Y22W (red squares) and GST-C1β (green triangles) to lipid vesicles containing 30 mol % PS and either 5 mol % DAG (filled symbols) or 1 mol % PMA (open symbols). Each data point represents the mean of triplicate experiments ± S.E. C, in vivo translocation of C1b domains to membranes. COS7 cells were cotransfected with PM-CFP and the indicated YFP-tagged C1b domain constructs: C1bβ (blue diamonds), C1β-β-Y22W (red squares), C1bα (green triangles), and C1bδ-W22Y (yellow circles). The relative translocation in response to UTP (100 μM) and PDBu (200 μM) treatment was calculated and plotted as a function of time. Data represent the average ± S.E. of 10–15 cells from three independent experiments. D, lysates from COS7 cells transfected with PKCβII, PKCβII-Y123W, or PKCβ were assayed for PKC activity in the presence of PS, DAG, and either Ca2+ (total PKC activity) or EGTA (calcium-independent PKC activity). Ca2+-independent PKC activity was calculated as a percent of total activity; total activity was comparable for both PKCβII constructs and typically slightly lower for the PKCβ construct. These data represent the relative activity of the overexpressed kinases in response to lipid cofactors in the absence of Ca2+. Data represent the average ± S.D. from three experiments.

Table 1

| Ligand | C1bβ | C1bβ-Y22W | C1bδ |
|--------|------|-----------|------|
| PS     | 780 ± 50 | 11.5 ± 0.5 | 35 ± 3 |
| PG     | 1690 ± 60 | 22 ± 1 | 130 ± 10 |
| PMA/PD  | 2.2 ± 0.2 | 1.9 ± 0.1 | 5.4 ± 0.5 |
| PS/PD  | 20 ± 6 | 2.9 ± 0.4 | 25 ± 3 |

of whether the ligand was DAG or PMA (Table 1). Trp-containing C1b 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). On the other hand, these proteins had no or 3-fold selectivity for PS for C1bβ-Y22W and GST-C1bδ, respectively, in the context of PMA-containing membranes. Taken together, these data reveal that Trp at position 22 increases the affinity of C1bδ for DAG-containing membranes, reduces selectivity between PMA and DAG, and increases DAG-dependent PS selectivity.

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 C1b 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 C1b construct; translocation to the plasma membrane was monitored as an increase in the ratio of FRET-based YFP emission: CFP emission (FRET ratio) (21).

UDP, 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 μM) resulted in an increase in FRET ratio, which was further increased to maximal translocation following addition of PDBu (200 μM). 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 cotransfected 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\(_{\beta1}\), PKC\(_{\beta1}\)-Y123W, and PKC\(_\delta\). Consistent with the standard model where Ca\(^{2+}\), DAG, and PS are required for full activation of cPKCs (27), PKC\(_{\beta1}\) 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\(_{\beta1}\) 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 pretargeting 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\(_\delta\) 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\(_{\beta1}\) or PKC\(_\delta\). PKC\(_\delta\) prelocalized to juxtanuclear membranes, while PKC\(_{\beta1}\) 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\(_{\beta1}\) did not cause any change in its localization, as PKC\(_{\beta1}\) 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 (C1z). 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-biding 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.

**FIGURE 2.** Residue 22 affects localization of the C1b domain (A) and full-length PKC (B). A, representative images of COS7 cells transfected with YFP-tagged C1b domains: C1b\(_\beta\) (top left), C1b\(_{\beta-Y22W}\) (top right), C1b\(_\delta\) (lower left), and C1b\(_{\delta-W22Y}\) (lower right), B, representative images of COS7 cells transfected with YFP-tagged full-length PKC: PKC\(_{\beta1}\) (top left), PKC\(_{\beta1}\)-Y123W (top right), PKC\(_\delta\) (lower left), and PKC\(_{\delta-W252Y}\) (lower right). Mutated residues correspond to position 22 of the C1b domain in the full-length protein. Data are representative of at least three independent experiments.

**FIGURE 3.** The ability of the C1 domain to bind DAG arises from modulation of the width and surface properties of the loops surrounding the hydrophobic DAG-binding cleft. Shown are a ribbon diagram overlay and molecular surfaces of C1b-PKC\(_\gamma\) (blue), C1b-PKC\(_\delta\) (green), and C1-PKC\(_\zeta\) (orange). Phorbol binds between the two loops at the top of the domain as indicated with an asterisk. The identity of residue 22 is marked with an arrowhead. Surface coloring scheme is as follows: blue, basic; red, acidic; yellow, polar; gray, nonpolar.
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 C1β lies in a very different orientation relative to the membrane compared with the indole ring of Trp-22 in C1βδ. 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 C1βδ is narrow and deep, while that of the non-DAG responsive C1βγ is wide and shallow. Thus, whereas the smaller DAG can bind well to C1βδ, only the larger phorbol esters can make hydrophobic contacts across the wide gorge of C1βγ.

Curiously, the C1 domain of PKCε has Trp at position 22, yet it still does not bind DAG. Rather, C1ε 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 C1βδ results in almost complete loss of binding to phorbol ester-containing membranes, while the reverse mutations in C1ε 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 responding to DAG alone, whereas cPKCs require the coordinated elevation of Ca²⁺. This cautions 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).

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