Interfacial Partitioning of a Loop Hinge Residue Contributes to Diacylglycerol Affinity of Conserved Region 1 Domains*

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*Running Title: Diacylglycerol affinity of C1 domains

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Background: The activity of protein kinase C isoenzymes (PKCs) is regulated by diacylglycerol (DAG).

Results: Aromatic residue that tunes DAG affinity influences the recruitment of the conserved region 1 (C1) regulatory domain to a membrane mimic.

Conclusion: Membrane pre-association step contributes to the affinity of C1 to diacylglycerol.

Significance: This work offers insight into the origin of differential DAG affinities in PKCs.

ABSTRACT

Conventional and novel isoenzymes of Protein Kinase C (PKCs) are activated by the membrane-embedded second messenger diacylglycerol (DAG) through its interactions with the C1 regulatory domain. The affinity of C1 domains to DAG varies considerably among PKCs. To gain insight into the origin of differential DAG affinities, we conducted high-resolution NMR studies of C1B domain from PKCδ (C1Bδ) and its W252Y variant. The W252Y mutation was previously shown to render C1Bδ less responsive to DAG (Dries et al. (2007) J. Biol. Chem. 282, 826-830) and thereby emulate the behavior of C1B domains from conventional PKCs that have a conserved Tyr at the equivalent position. Our data revealed that W252Y mutation did not perturb the conformation of C1Bδ in solution but significantly reduced its propensity to partition into a membrane-mimicking environment in the absence of DAG. Using detergent micelles doped with a paramagnetic lipid, we determined that both the residue identity at position 252 and complexation with diacylglycerol influence the geometry of C1Bδ-micelle interactions. In addition, we identified the C-terminal helix α1 of C1Bδ as an interaction site with the head groups of phosphatidylserine, a known activator of PKCδ. Taken together, our studies (i) reveal the identities of C1Bδ residues involved in interactions with membrane-mimicking environment, DAG, and phosphatidylserine, as well as the affinities associated with each event, and (ii) suggest that the initial ligand-independent membrane recruitment of C1B domains, which is greatly facilitated by the interfacial partitioning of Trp252, is responsible, at least in part, for the differential DAG affinities.

INTRODUCTION

The objective of this work was to determine how conserved region 1 (C1) domains interact with membrane-like hydrophobic environment and lipophilic cofactors that activate the parent enzyme, PKC. PKC is involved in a diverse web of cell signaling events that lead to cellular processes such as division, migration, and apoptosis (1-3). The timing of PKC activation in the cell is tightly controlled by the second messenger signals, which relieve the auto-inhibitory interactions within the enzyme by binding to its regulatory domain (4,5).
Conventional (α, βI, βII, and γ) and novel (ε, δ, θ, and η) isoforms of PKC are activated by the second messenger DAG (6,7); conventional isoforms additionally require Ca\(^{2+}\) for membrane recruitment. The interaction with the membrane-embedded DAG is mediated by the C1 regulatory domain (8-11). Little is known about the affinity or identity of C1 residues that interact with the membrane components during the initial membrane-association step. The studies presented here address the question of DAG-independent interactions of C1 domains with a membrane-mimicking environment and their influence on DAG affinities.

In novel and conventional PKCs, the C1 regulatory domain comprises two highly homologous tandem lipid-binding modules referred to as C1A and C1B. C1A and C1B have different intrinsic affinities to DAG and phorbol esters (PE), which are widely used as pharmacological and research tools to study the role of PKC in tumorigenesis. The differential ligand affinities of C1A and C1B were reported in the context of isolated domains (10,12,13) and full-length enzymes (9,14-16). DAG affinity across the PKC isozymes varies as well: the C1 domain from a novel isozyme, PKCδ, has a significantly higher affinity to DAG-containing membranes than the C1 domain from a conventional isozyme, PKCβII (17). It has been suggested that novel PKC isoforms “compensate” for their lack of Ca\(^{2+}\)-dependent membrane-binding function through enhanced affinity of their C1 domains to DAG compared to conventional isoforms (17).

The affinity of PKCs to DAG has important implications for the cellular localization of PKC isoforms and selectivity of their response (18). However, the origin of differential DAG affinities remains obscure. All DAG-responsive C1 domains have a similar architecture: a ~50 amino acid core that folds into two β sheets and a C-terminal α helix, α1 (19-23). The tertiary structure is stabilized by two structural Zn\(^{2+}\) ions, each coordinated by three Cys and one His side-chains, as shown for the C1B domain from PKCδ (C1Bδ) in Figs. 1A and C. The only high-resolution structure of ligand-bound C1, the C1Bδ-phorbol 13-acetate complex (19), revealed that the ligand-binding pocket is formed by two loops between the β strands 1-2 (β12 loop) and 3-4 (β34 loop) (Fig. 1). Surprisingly, the ligand – which is considered to be a “partial” PKC activator (24) compared to DAG and more hydrophobic PEs – does not make any sidechain-specific contacts with the protein, forming hydrogen bonds with the backbone groups instead (Fig. 1B). From this structure alone, it is unclear how the residue identities lead to dramatic changes in ligand binding affinities and PKC isoform activities.

A critical component that is usually omitted from high-resolution structural studies of C1 domains is membranes or membrane mimics. We previously reported a micelle system that enabled us to obtain residue-specific information about DAG and PE binding to the C1B domain from PKCα (25). One component of this micelle system is a detergent bearing the phosphatidylserine (PtdSer) head group. The role of PtdSer as a specific phospholipid activator of PKCs was discovered in the late 1980s (26-28). The activating power of PtdSer stems from its ability to enhance the binding affinity of PKCs to membrane-embedded ligands, such as PEs and DAG. In conventional PKC isoforms, both C1 and C2 domains contribute to the interactions with PtdSer, the latter binding PtdSer in a Ca\(^{2+}\)-dependent manner (14,29-31). In contrast, novel isoforms, such as PKCδ, interact with PtdSer solely through their C1 domains. This has been demonstrated – in the presence of DAG/PE – for full-length PKCδ (16) and isolated C1B domains (32-34). Despite the significance of PtdSer in the activation of PKCs, the identities of residues involved in these interactions remain unknown.

In this work, we applied high-resolution NMR methods to the wild-type C1Bδ (wtC1Bδ) and its diagnostic variant W252Y to obtain residue-specific information about their interactions with lipophilic effectors. The properties of C1Bδ, which differs from the conventional C1B domains in amino acid sequence (58% identity with C1Bα and C1Bβ), higher intrinsic affinity to DAG, and position in the primary structure of the parent enzyme, have enabled us to probe for the first time the ligand-independent interactions of C1 domains with membrane mimics. To mimic the membrane environment, we used a micelle system that contained a PtdSer component and faithfully
reproduced the pattern of DAG affinities previously measured in lipid bilayers (35). Our findings offer insight not only into the PKCδ-specific interactions with membrane-like hydrophobic environment and DAG, but also into the origin of differential DAG affinities in conventional and novel PKC isozymes.

**EXPERIMENTAL PROCEDURES**

**Preparation of C1Bδ samples** – The DNA sequence of the C1Bδ domain from *Rattus norvegicus* was amplified by PCR using the cDNA clone of PKCδ (Open Biosystems) as a template. A 53-residue construct of C1Bδ (residues 229-281) was subsequently cloned into a pET-SUMO expression vector (Invitrogen). W252Y mutation was introduced into the plasmid using the QuikChange® Site-Directed Mutagenesis kit (Stratagene) and appropriate primers. Both C1Bδ variants were over-expressed and purified as described previously for C1Bα (25). Isotope labeling with 13C and 15N was carried out in M9 minimal media using [U-13C] glucose and [U-15N] ammonium chloride as sole carbon and nitrogen sources, respectively.

**Assignment of NMR resonances** – NMR experiments were carried out on a 14.1 T VNMRS instrument (1H Larmor frequency of 600 MHz) equipped with a room-temperature triple-resonance probe. The temperature was 25 ºC, as calibrated with methanol. The NMR sample contained 1 mM [U-15N, 13C] enriched apo wtC1Bδ in the NMR buffer: 10 mM [2H-4] imidazole (Cambridge Isotopes) at pH 6.5, 150 mM KCl, 8% 2H2O, 1 mM tris(2-carboxyethyl)phosphine, and 0.02% NaN3. Resonance assignments for apo wtC1Bδ were obtained from the following 3D NMR experiments: CBCA(CO)NH, HNCACB (36), H(CC0)NH (37), and 15N-edited NOESY. NMRPipe (38) and SPARKY 3 (39) software packages were used for data processing and assignment, respectively. HNCACB and CBCA(CO)NH NMR experiments were also conducted on the sample containing 0.5 mM wtC1Bδ and 1 mM 1,2-dioctanoyl-sn-glycerol (DOG) in 100 mM mixed micelles (3:7 molar ratio of 1,2-dihexanoyl-sn-glycero-3-[phospho-L-serine] (DPS) and [3H38] n-dodecylphosphocholine (DPC)), to verify the protein assignments in the DOG-complexed state.

**DOG and PDBu binding experiments** – DOG or the short chain phorbol ester, phorbol-12,13-dibutyrate (PDBu, Sigma-Aldrich) were dissolved in [2H6] DMSO (Cambridge Isotopes) and added stepwise into 0.1 mM wtC1Bδ or W252Y pre-incubated with 10 mM mixed micelles (DPS/DPC, 3:7 molar ratio). 15N-H HSQC spectra were collected on a 14.1 T Varian INOVA NMR spectrometer at each concentration of the ligand. DMSO concentration was less than 7% (vol/vol) at the highest ligand concentration. For W252Y, the binding curves were constructed by plotting the overall change in chemical shift Δ against the total DOG concentration, L0. Δ was calculated according to the following equation (40):

\[
\Delta = (\Delta \delta^2 + (0.152 \Delta \delta_N)^2)^{1/2}
\]

The curves were fit with the single-site binding equation (41):

\[
\Delta = (\Delta \delta_{PL} / 2P_0) \times [K_d + P_0 + L_0 - ((K_d + P_0 + L_0)^2 - 4P_0 L_0)^{1/2}]
\]

where ΔδPL is the difference between chemical shifts of the apo and bound states, and P0 is the total protein concentration.

The binding of DOG to wtC1Bδ was also detected by following the change in the fluorescence emission of Trp252. The experiments were carried out on ISS Koala fluorometer (ISS, Champaign, IL), with excitation at 295 nm and detection with a 335 nm cut-on filter. The total protein concentration was 0.5 µM in the “NMR buffer”, where imidazole was replaced with 10 mM 2-(N-morpholino)ethanesulfonic acid (Fisher Scientific). As observed previously for C1Bα (25), the effective protein concentration in solution was 50% of the total due to adsorption on cuvette walls. After each subsequent addition of DOG, the fluorescence emission spectrum was recorded on a protein sample and a “blank” sample containing...
the same components with the exception of the protein. The fluorescence intensities were subtracted to correct for any change due to DOG/DMSO addition that did not arise from protein binding.

The fraction of DOG-complexed wtC1Bδ was calculated as ΔF/F0, where ΔF is the absolute value of the intensity change corrected for protein dilution during the titration, and F0 is the fluorescence intensity before titrating DOG. The experiments were performed in triplicate.

**Micelle binding experiments** – All NMR measurements were conducted on an 18.8 T Bruker Avance III instrument (¹H Larmor frequency 800 MHz) equipped with a cryogenically cooled triple-resonance probe. The samples contained 0.1 mM [U-¹⁵N]-enriched C1Bδ (wt or W252Y) in the “NMR buffer” with a KCl concentration of 20 mM. Micelle binding was detected using the fHSQC experiment (42). The binding experiments were carried out with the following ligands: (i) pure DPC micelles, (ii) DPS, titrated into the binary C1Bδ-DPC micelle complex, and (iii) mixed DPS/DPC micelles having 3:7 molar ratio of the detergent components. The detergent concentration ranged from 3.0-100 mM in (i), 1.3-178 mM in (ii), and 5.0-200 mM in (iii). The total detergent concentration in experiments (i) and (iii) was converted to micelle concentration using an aggregation number of 56. The aggregation number was calculated from the translational diffusion coefficients that we measured in a set of pulsed-field gradient NMR experiments (43), and is consistent with previous findings (44). The binding curves were constructed by plotting the residue-specific chemical shift changes Δ against the micelle concentration in (i) and (iii), and the total DPS concentration in (ii). The apparent dissociation constants of the binary protein-micelle complexes were obtained from the fit of the binding curves to Eq. 2.

**RESULTS**

W252Y mutation decreases the affinity of C1Bδ to DOG without significant perturbations of the structure – It has been demonstrated that the identity of residue at position 22 (or 252 if using the numbering scheme of full-length PKCδ) has a profound influence on the affinity of C1 domains to DAG (35). This residue is an invariant Tyr in C1B domains of conventional PKCs, and is Trp in all other C1 domains of conventional and novel PKCs (45). In the C1B domains of α and β isoforms, the mutation of Tyr to Trp increased the DAG affinity 31-fold in large unilamellar vesicles (35), and >100-fold in micelles (25). Similarly, the mutation of native Trp252 to Tyr made C1Bδ less responsive to DAG in vivo, as demonstrated by the membrane translocation and localization experiments for both isolated domain and full-length PKCδ (35).

A comparison of the ¹⁵N-¹H HSQC spectra of the wtC1Bδ and the W252Y variant (Fig. 2) indicates that the effect of mutation on the protein conformation is minor. This is further illustrated by the chemical shift perturbation (CSP) analysis: the difference between wtC1Bδ and W252Y chemical shifts does not exceed 0.15 ppm (inset in Fig. 2). One distinct feature of the C1Bδ ¹⁵N-¹H HSQC spectrum is that the cross-peaks for some residues of the loops β12 and β34 are missing, among them the backbone ¹H,¹⁵N resonance of the mutation site W252. This indicates that the loops undergo a conformational exchange process that is intermediate on the NMR chemical shift timescale. W252Y mutation alters the features of this dynamic process, as is evident from the reappearance of T242, Y252, G253, K256, Q257, and G258 in the spectra.
To identify the specific residues involved in diacylglycerol binding, we conducted NMR-detected titration experiments of C1B\(\delta\) with DOG, a short-chain diacylglycerol. In the absence of membrane mimics, W252Y showed no evidence of binding up to a 20-fold molar excess of ligand. wtC1B\(\delta\) precipitated upon DOG addition in what could be a combined effect of ligand binding and protein aggregation. These results clearly demonstrate that the use of membrane-mimicking environment is absolutely essential for the functional and biophysical studies of these membrane-targeting domains. To mimic the membrane environment, we used mixed micelles comprising DPS/DPC with a molar ratio of 3:7. Upon addition of DOG to the micelle-containing sample, the wtC1B\(\delta\) residues involved in ligand binding show slow-to-intermediate exchange behavior, where the cross-peaks corresponding to the ternary protein-micelle-DOG complex appear at sub-stoichiometric concentrations of ligand and gradually build up until reaching saturation (Fig. 3A). NMR data in this exchange regime are not suitable for quantitative analysis. Instead, the change in the intrinsic fluorescence of Trp252 was used to construct the DOG binding curve (Fig. 3C). Even at sub-micromolar concentrations of C1B\(\delta\), the curve is typical for the high-affinity binding regime. The fluorescence data enabled us to put an upper limit estimate of < 0.2 \(\mu\)M on the dissociation constant, \(K_d\). In contrast to the wt C1B\(\delta\), DOG binding to the W252Y variant is in the intermediate-to-fast regime (Fig. 3B). We constructed the binding curves based on the dependence of the chemical shift changes on the DOG concentration (Fig. 3D). Fitting the data to Eq. 2 produced a \(K_d\) of 5.2±0.5 \(\mu\)M, which means that the mutation of Trp252 to Tyr results in a >26-fold decrease in the DOG binding affinity.

In addition to DOG experiments, we conducted a series of binding experiments with PDBu, a tumor-promoting phorbol ester widely used in PKC functional assays. In contrast to DOG, wtC1B\(\delta\) and the W252Y variant have a very similar response to PDBu binding, which occurs in the slow-to-intermediate exchange regime (data not shown). This is consistent with the previous results showing that although the W252Y mutation caused a 10-fold decrease in membrane translocation in response to diacylglycerol, it had no effect on the PDBu-stimulated membrane translocation of C1B\(\delta\) (35).

We conclude that our micelle system is a reliable membrane mimic to study the molecular details of the C1B\(\delta\)-ligand interactions.

The CSP analysis for the pairs of apo- and micelle/DOG-bound states revealed that in both C1B\(\delta\) variants the ligand binding influences essentially the same set of residues to a similar extent. The only significant differences in chemical shifts between the ternary C1B\(\delta\)-DOG-micelle complexes were observed for the residues surrounding the mutation site: L250, L251, and G253. These local changes provide little insight into the origin of increased DOG affinity in Trp-containing C1B\(\delta\) domains.

C1B\(\delta\) partitions into micelles in a ligand-independent manner – It became evident in the course of the DOG binding studies that addition of just mixed micelles results in significant changes of chemical shifts in both C1B\(\delta\) variants (compare black and blue spectra of Figs. 3A and B). This means that C1B\(\delta\) can partition into the hydrophobic micellar environment even in the absence of DOG. While the membrane association of C1 domains has been hypothesized to be the initial step required before DAG binding (46), very little is known about this process. We therefore sought to systematically characterize the ligand-independent interactions of C1B\(\delta\) with micelle components. The advantage of using solution NMR methods for these studies is that the information about binding can be obtained in the residue-specific manner.

In the first set of experiments, we tested the binding of [U-\(^{15}\)N]-enriched wtC1B\(\delta\) to micelles composed of pure DPC, a zwitterionic detergent. The total concentration of DPC was kept above the 1.1 mM critical micellar concentration and ranged from 3 to 100 mM. The formation of the binary protein-micelle complex was monitored in a series of \(^{15}\)N-\(^{1}\)H HSQC spectra. The kinetics of binding falls into fast and intermediate-to-fast exchange regime on the NMR chemical shift timescale. All residues that experience significant chemical shift perturbations upon interacting with micelles belong to either \(\beta_{12}/\beta_{34}\) loops or their hinges (Fig. 4A). This is especially pronounced for the loop tips (S240 of \(\beta_{12}\) and V255 of \(\beta_{34}\)), and the N-terminal \(\beta_{34}\) hinge (L250 and the sidechain H-
Interestingly, the dynamic nature of the ligand-binding loops is preserved in the protein-micelle complex, because only three (M239, W252, and G258) out of the twelve exchange-broadened residues in the apo wtC1Bδ reappear in the spectra upon micelle saturation.

For ten residues of C1Bδ, we constructed the binding curves by plotting the chemical shift change versus the total concentration of DPC micelles. The total concentration of DPC was converted into micellar concentration assuming 56 DPC molecules per micelle. The binding curves were fit globally with Eq. 2 to obtain the effective dissociation constant $K_d$ of $22.4 \pm 1.0 \, \mu M$ (Fig. 4B). Our results demonstrate that the hydrophobicity of the loop region, which constitutes about a quarter of the protein, is sufficient to drive the ligand-independent partitioning of C1Bδ into a membrane mimic.

C-terminal helix $\alpha 1$ of C1Bδ responds to the presence of PtdSer – To determine the influence of PtdSer on the interaction of C1Bδ with a membrane-mimicking environment, we tested the binding of [U-$^{15}$N]-enriched wtC1Bδ to mixed DPS/DPC micelles. The chemical shift perturbation analysis revealed significant differences with the DPC-only data (compare Figs. 4A and C). While the same subset of loop residues is affected by the interaction, the perturbation in helix $\alpha 1$ that comprises residues 270-275 is significantly more pronounced in the mixed micelle sample. In particular, there are three residues that have higher than average CSP values: H270, K271, and E274.

Quantitative analysis of the C1Bδ binding curves revealed heterogeneous behavior. The group that includes the most DPC-responsive residues has high affinity to mixed micelles with an apparent $K_d$ of $9.0 \pm 1.0 \, \mu M$. The group comprising helix $\alpha 1$ residues has a low affinity, with an apparent $K_d$ of $144 \pm 1.0 \, \mu M$. Representative binding curves from both groups are given in Fig. 4D. This behavior is in contrast with that observed in DPC-only micelles, where all binding curves could be fit with the same global $K_d$. We conclude that (i) presence of the PtdSer component enhances the apparent affinity of wtC1Bδ to micelles ~2.5-fold, as reported by the loop/hinge residues; and (ii) $\alpha 1$ helix participates in the interactions with PtdSer.

To ascertain the direct nature of C1Bδ-DPS interactions, we prepared a binary wtC1Bδ-DPC complex under conditions where the protein is fully micelle-bound. We then added the DPS stepwise to this complex and monitored the changes in NMR spectra. Helix residues H270 and E274 experience large chemical shift perturbation, fully consistent with the CSP pattern in mixed micelles. K271 is exchanged-broadened and could not be used for analysis. One interesting observation is that upon increasing the DPS concentration the loop residue cross-peaks move in the direction of their position in the apo state of wtC1Bδ (data not shown). We speculate that this behavior could represent either the re-orientation of wtC1Bδ in DPC micelles upon DPS binding, and/or a gradual redistribution of the protein between DPC and DPS micelles, with the latter primarily interacting with helix $\alpha 1$ rather than loop regions.

A model of the C1Bδ-mixed micelle interactions consistent with our NMR data is shown in Fig. 4E. In this model, C1Bδ can bind to both DPS/DPC (event 1) and DPC-only (event 2) sites. A large difference between the apparent $K_d$ values of loop/hinge and $\alpha 1$-residues, 9 and 144 $\mu M$ (Fig. 4D), implies that the plateau region for the latter is reached at higher concentrations of mixed micelles. This scenario would not be possible if C1Bδ interacted exclusively with the micelle-incorporated DPS. One explanation is that C1Bδ can additionally recruit DPS monomers from solution (event 3). Analysis of the pulsed field gradient NMR data using $^1$H resonances of DPS as reporters revealed that in the mixed micelle system there is indeed a small fraction of monomeric DPS.

Effect of PtdSer on the ternary C1Bδ-DOG-micelle complex – We then asked if DOG-bound C1Bδ interacts with DPS in a similar manner. We compared the DOG-saturated C1Bδ spectra in two different micellar environments: DPC-only and mixed DPS/DPC with the 3:7 molar ratio. Compared to the apo state of the protein, residues that belong to the $\beta 12/\beta 34$ loops and their hinges show large chemical shift perturbations in response to binding DOG and DPC-only micelles (solid arrows in Fig. 5A). The same subset of
residues, including the sidechain of W252, is only moderately influenced by the micelle-incorporated DPS (dashed arrows in Fig. 5A). In contrast, α1 residues H270, K271, and E274 are significantly affected by DPS but not DPC. Of the three, H270 and K271 (inset of Fig. 5A) experience the largest absolute chemical shift changes. This pattern is fully consistent with C1Bδ behavior in micelles without DOG (Fig. 4).

In addition to H270 and K271, the N- and C-terminal hinges of loop β34, W252 and Q257-G258, are influenced by the presence of DPS. Q257-G258 is a conserved “QG” motif present in all C1 domains (45). Mutagenesis studies indicate that Q257 in particular plays an important role in protein-ligand interactions (47,48). The QG motif is highly dynamic in the apo state of the parent protein, as evidenced by its exchange-broadening in the NMR spectrum of apo wtC1Bδ. Upon either DOG or PDBu binding to C1Bδ in the micellar environment, the cross-peaks of the QG motif reappear in the spectra, suggesting that loop dynamics is either attenuated or its timescale changes to a faster one. Loop β12 is not significantly influenced by the presence of DPS.

In summary, our NMR data support the existence of a PtdSer interaction site on the C1Bδ domain that includes the residues of the α1 helix: H270, K271, and possibly E274. H270 and K271 are in close proximity to the N- and C-terminal hinges of ligand-binding loop β34.

**The identity of residue 252 tunes the affinity of C1Bδ interactions with a membrane mimic —** The unexpected finding that C1Bδ efficiently partitions into the micellar environment in the ligand-independent manner prompted us to examine the role of residue 252 in this process. We hypothesized that ligand-independent interactions of C1Bδ contribute to the differences in the apparent DOG affinities that we observed for the wild-type protein and its W252Y variant.

We conducted the NMR-detected titration experiments of the [U-15N] enriched W252Y C1Bδ variant with mixed DPS/DPC micelles. Note that because the W252Y mutation changes the dynamic properties of apo C1Bδ, we obtained a more extensive “coverage” of the loop region in the CSP plot (Fig. 6A) compared to the wild-type protein (Fig. 4C). Mapping the perturbed residues onto the three-dimensional structure highlights the same three regions as in the wtC1Bδ: two loops/hinges, and the early part of the α1 helix. The largest chemical shift perturbation is experienced by G253, which is located near the tip of the β34 loop. Based on the NMR chemical shift data, we constructed the binding curves for all micelle-responsive residues of W252Y. This analysis revealed the presence of two sites with distinct affinities (Figs. 6B and 6C). The apparent affinity of loop regions to the mixed micelles is 134±7 μM, which is approximately 15-fold higher than that observed for the wtC1Bδ. The three residues of the α1 helix, H270, K271, and E274, were globally fit to obtain an apparent Kd of 364±34 μM. This value is ~2.5-fold higher than that of the wtC1Bδ.

A summary of the apparent dissociation constants obtained from the ligand and micelle-binding experiments is given in Fig. 6D. These data show that the identity of the residue at position 252 not only controls DOG affinities, but also influences the ligand-independent partitioning of wtC1Bδ into a membrane-mimicking hydrophobic environment.

**Depth of C1Bδ insertion depends on residue identity and the presence of ligand —** We used the paramagnetic relaxation enhancement (PRE) of N-H groups to evaluate how the residue identity at position 252 and interactions with DOG influence the depth of C1Bδ insertion into mixed micelles. A lipid bearing a paramagnetic doxyl tag, 14-doxyl PC, was introduced into the mixed micelles during the preparation step to give ~0.7 14-doxyl PC molecules per micelle. The unpaired electron of the doxyl group enhances the transverse relaxation rates of proximal nuclear spins, primarily 1H, resulting in broadening of the corresponding cross-peaks with the concomitant reduction in their intensities. The residue-specific PRE values were calculated as the intensity ratios of the N-H cross-peaks of C1Bδ bound to the paramagnetic and diamagnetic preparations of DPS/DPC micelles, $I_{param}/I_{dia}$. A total of four experiments were carried out: with and without DOG, for two C1Bδ variants. For consistency, the same preparation of diamagnetic and paramagnetic micelles was used for all experiments.

A common feature of all four data sets is that the loop regions experience a clear gradient of insertion depth (Figs. 7A and B). Residues in the...
vicinity of loop tips, e.g., S240 in β12 and L254/V255 in β34, are completely broadened due to PRE. The PRE becomes progressively smaller toward the loop hinges and is virtually non-existent for the N- and C-terminal regions of the C1Bδ variants. Another segment that shows an appreciable PRE value is the 3 amino-acid stretch, δC1Bexistent for the N- and C-terminal regions of the α1 helix. α1 itself shows a very weak gradient of insertion depth, from low (His270) to very low (Lys275). The PRE results are in agreement with our CSP data (Figs. 4C and 6A) that implicate the entire loop region in the interaction with the hydrophobic micellar environment, and the early part of α1 in surface interactions with the PtdSer headgroup.

DOG influences the geometry of C1Bδ-micelle interactions. The PRE data for both C1Bδ variants show the same trend: a decrease in insertion depth due to DOG binding, as reported by the loop hinge regions (compare green and black bars in Figs. 7A and 7B). The effect of DOG is most clearly seen in the difference plot of W252Y PRE values showing the positive Δ(Ipara/I dia) values for the C-terminal hinge of loop β21 (F243 and I244) and the N- and C-terminal hinges of loop β34 (e.g., L251 and L259) (Fig. 7B). Color-coding residue-specific PRE values and mapping them onto the C1Bδ structure highlights the PRE differences at the level of individual residues (Fig. 7C).

A pairwise correlation of the PRE values between the W252Y variant and wtC1Bδ revealed a number of “outliers” in the DOG-free and DOG-complexed states (Fig. 7D). These outliers represent residues with significantly different PRE values. For the DOG-free domains, all outliers fall in the upper diagonal part of the plot, indicating that their insertion depth is larger in the wtC1Bδ than in the W252Y variant. This trend is reversed in the DOG-complexed state, where the outliers consistently fall in the lower diagonal part of the plot. Of particular note is the backbone N-H group of the mutation site, 252, with the PRE values of 0.09 and 0.33 in the W252Y and wtC1Bδ, respectively. With a notable exception of H270, which is implicated in the interactions with PtdSer, the outliers belong to the hinge regions of loops β12 and β34. In summary, our PRE data revealed that both interactions with DOG and residue identity at position 252 influence the insertion depth and most likely the orientation of C1Bδ in the micellar environment.

**DISCUSSION**

**Trp versus Tyr in C1 domains** – In diacylglycerol-responsive C1 domains, the residue at position 252 or equivalent is either a Trp or Tyr (45). The residue identity has a profound effect on DAG sensitivity of C1 domains (35) but does not result in any significant structural perturbation as a result of Trp→Tyr mutation in C1Bδ (Fig. 2) or Tyr→Trp mutation in C1B from PKCα (C1Bα) (25). We speculate that both the position of this residue in the 3D domain structure and the special properties of Trp sidechain contribute to the functional differences of Trp- and Tyr-containing C1 domains.

Position 252 corresponds to the N-terminal hinge of the β34 ligand-binding loop (Fig. 1). The distance between the indole nitrogen of the W252 residue and the methyl groups of L254 at the tip of the β34 loop is between 12 and 13 Å. Comparison of these distances with the experimentally determined membrane geometries (49-51) – ~15 Å for the half-width of the hydrocarbon core and ~5-7 Å for the headgroup region – suggests that an aromatic residue at position 252 is likely to be localized at the interfacial region that consists of lipid/detergent head-groups and water molecules (52).

The propensity of both Tyr and Trp to partition into the membrane headgroup region and thereby anchor their host proteins is well established (53-56). We demonstrated in this work that even in the absence of DOG the replacement of Trp with Tyr in C1Bδ results in a 15-fold decrease in the apparent affinity for micelles (Fig. 6D). Aside from the protein-specific effects, such as modulation of the backbone dynamics, what are the differences between the Trp and Tyr sidechains that may contribute to the differential affinities?

Both side-chains are amphiphilic and have functional groups that can participate in hydrogen-bonding interactions: Nε1-Hε1 (Trp) and Oη-Hη (Tyr). However, Trp and Tyr side-chains differ in hydrophobicity, aromaticity, and the magnitude of the electric dipole moment. According to the White-Wimley hydrophobicity scale (57,58), Trp
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is the most hydrophobic amino acid, with a ~2-fold difference in the free energies of water-octanol and water-bilayer transfer compared to Tyr (59). This means that hydrophobic effect (i.e. solvent exclusion) will make a larger favorable contribution to the free energy of binding in wtC1Bδ compared to W252Y. The extended aromatic system of Trp → two fused rings, as opposed to one ring in Tyr → is likely to facilitate the cation-π interactions (60) with positively charged choline groups of the lipids and detergents (61). Differences in the electric dipole moments of indole and phenol moieties (2.1 D and 1.2 D, respectively (56,62)) may contribute to the more favorable charge-dipole and dipole-dipole interactions of the Trp side-chain with the complex electrostatic environment of the headgroup regions and the associated water.

Relating our results to the mechanism of C1 recruitment to membranes – The functional role of ligand-independent interactions of C1 domains with membranes is underscored by a recent single-molecule study that implicated the C1A domain in the formation of a membrane-bound “pre-DAG intermediate” of PKCα (63). Newton’s group conducted an elegant mechanistic study of the association of C1B from PKCβII (C1Bβ) domain with membrane-embedded ligands, using a combination of stopped-flow and equilibrium membrane binding experiments (46). A three-step mechanism was proposed: initial recruitment of C1 domains to membranes, two-dimensional search for a membrane-embedded ligand, and ligand binding.

Our data on the ligand-independent partitioning of C1Bδ into the membrane-mimicking environment (Figs. 4, 6, and 7) provide direct evidence for the first step of this mechanism. High-resolution NMR methods enabled us to identify, for the first time, specific residues in C1Bδ that are involved in this process. We found that, irrespective of the residue identity at position 252, the C1Bδ interaction site with micelles is rather extensive and encompasses the entire loop region.

The second step of the proposed mechanism involves a two-dimensional search for membrane-embedded diacylglycerol. A similar pattern of wtC1Bδ/W252Y diacylglycerol affinities in our micelle system (Figs. 3 and 6D) and bilayers (35) suggests that the same factors are at play. We conclude that ligand-independent interfacial partitioning of C1Bδ, which is facilitated by the Trp at position 252, contributes to the enhancement of apparent diacylglycerol affinity in the wtC1Bδ compared with its Tyr-containing variant.

Novel versus conventional C1 B domains – Based on the mechanistic study of C1Bβ (46), we expected the ligand-independent interactions of C1Bδ with a membrane mimic to be weak. In our previous NMR investigation of C1Bα and its Tyr→Trp variant, the kinetics of binding to DPS/DPC micelles was intermediate on the chemical shift timescale, thereby precluding the affinity measurements (25). In this work, we found that both wtC1Bδ and W252Y have a modest and quantifiable affinity to mixed micelles. This prompted us to compare the properties of the two C1B domains that were used in the above-mentioned studies and C1Bδ.

Kyte-Doolittle (64) hydropathy plots of the three C1B domains are very similar, with the exception of the C-terminal helix α1 (Fig. 8). This region has a lower hydropathy value and hence is more hydrophilic in C1Bδ than it is in C1Bα and C1Bβ. At the level of primary structure, this behavior mostly stems from the differences at positions 273 and 274: charged residues, Arg and Glu, in C1Bδ and hydrophobic residues Val and Ile(Met) in C1Bα(β). The backbone N-H groups of α1 experience a very weak PRE effect, indicating that α1 is positioned outside the micelle (Figs. 7A and B). It is plausible that the side-chains of charged residues, which make up most of the α1 in C1Bδ, interact with the polar groups of the micelle or membrane surface and thereby contribute to the free energy of binding.

Evidence for the PtdSer interaction site – We observed distinct chemical shift changes in the α1 region upon incorporating the PtdSer component into our micelle system. PtdSer recognition sites in proteins are notoriously difficult to identify due to the absence of a well-defined binding motif (65). NMR methods used in this study enabled us to evaluate the effect of PtdSer on C1Bδ in a residue-specific manner. The pattern that emerged is similar for the DOG-free and DOG-bound C1Bδ. Three α1 residues experience large-to-
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moderate chemical shift perturbations when PtdSer is present either as a micelle component or added to the DPC-bound domain: H270, K271, and E274. Among these three, a positively charged residue at position 271 is highly conserved among C1 domains (45). All three side-chains are located on the same face of α1, suggesting that the early part of the helix may be involved in direct electrostatic interactions with the PtdSer head-groups. Indeed, mutation of positively charged residues at positions equivalent to 270 and 271 in the C1A domain of PKCα leads to decreased PtdSer-dependent kinase activity (31).

Depth of insertion – Recent comparative analysis of the polarities of membranes and membrane protein structures revealed that the aromatic ring of Trp is positioned 3-4 Å closer to the membrane center than Tyr (66). We found that Trp at position 252 facilitated deeper insertion of wtC1Bδ into the micellar environment compared to the W252Y variant, while the pattern was reversed in the respective DOG complexes (Fig. 7D). The implication of these findings for the membrane recruitment of PKC is that the residue identity at position 252 can influence the geometry of C1Bδ-membrane interactions, whether in the apo state during the initial membrane pre-association step (vide supra) or upon binding to the membrane-embedded diacylglycerol. In addition, interactions with diacylglycerol are likely to re-position the domain in the membrane more towards the headgroup region (difference plot of Fig. 7B). Even subtle changes in the geometry of C1Bδ-membrane interactions will be transduced to the neighboring domains in the parent PKC via the linker regions. This in turn may influence the overall activation of PKC, by modulating the affinities of these domains to membrane-embedded ligands and/or other interacting protein partners.

Conclusions – Our data suggest that the ligand-independent membrane pre-association step, which is greatly facilitated by the Trp residue at position 252 or equivalent, contributes to the affinity of C1 domains to membrane-embedded DAG. Special properties of the Trp sidechain make it an ideal residue to anchor the ligand-binding loops of C1Bδ in the complex environment of the head-group region and position the domain appropriately for binding to DAG. In addition to the loop regions, the C-terminal helix α1 is likely to play a role in positioning the C1 domains, through its electrostatic interactions with the phosphatidyl-serine and -choline head groups of the membrane components. Understanding the geometry of C1 interactions with membranes at the atomic-level will require either implementation of docking approaches (67) or full structure determination in NMR-compatible membrane mimics.
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FOOTNOTES

1The abbreviations used are: PKCs, protein kinase C isoenzymes; DAG, diacylglycerol; C1, conserved region 1; PE, phorbol esters; C1Bδ, C1B domain from PKCδ; PtdSer, phosphatidylserine; DOG, 1,2-dioctanoyl-sn-glycerol; DPS, 1,2-dihecanoyl-sn-glycerol; DPC, n-dodecylphosphocholine; HSQC, hetero-nuclear single-quantum coherence; PDBu, phorbol-12,13-dibutyrate; PRE, paramagnetic relaxation enhancement; 14-doxyl PC, 1-palmitoyl-2-stearoyl-14-doxyl-sn-glycero-3-phosphocholine; CSP, chemical shift perturbation; C1Bα, C1B from PKCα.

FIGURE LEGENDS

FIGURE 1. (A) Structure of C1Bδ complexed to a water-soluble phorbol ester, phorbol-13 acetate (PDB ID 1PTR (19)). The loop regions, β12 (residues 237-242) and β34 (residues 252-257), are highlighted in green. α1 is a short α-helix comprising residues 270-275. Two structural Zn²⁺ ions are shown as gray spheres. (B) Expansion of the C1Bδ ligand-binding site showing the hydrogen bonds between phorbol-13 acetate (P13A) and the backbone atoms of C1Bδ. The sidechain of W252 is not involved in direct interactions with the ligand. (C) The primary structure of C1Bδ with W252 and Zn²⁺-coordinating residues highlighted with orange and yellow, respectively. β12 and β34 loops are underlined and highlighted with green.

FIGURE 2. W252Y mutation does not significantly perturb the conformation of C1Bδ in solution. Overlay of the ¹⁵N-¹H HSQC spectra of the wtC1Bδ (black) and W252Y (red), with resonance assignments shown next to the corresponding cross-peaks. Peaks that are exchange-broadened in the wtC1Bδ spectrum, but appear in the W252Y spectrum are in boldface and underlined. N237 and K271 resonances, indicated with “X” symbols, are below the contour level threshold. Aliased Arg side-chain peaks are marked with asterisks. Inset: CSP value Δ plotted as a function of the primary structure. The mutation site is indicated with an arrow. Exchange-broadened residues and Pro241 are marked with asterisks.

FIGURE 3. W252Y variant binds DOG with lower affinity than wtC1Bδ. The expansions of the ¹⁵N-¹H HSQC spectra are shown at increasing concentrations of DOG for the 100 µM wtC1Bδ (A) and W252Y (B) in the presence of 10 mM DPS/DPC. The spectra of apo proteins in the absence of micelles are in black. (C) DOG-binding curve of C1Bδ obtained from the fluorescence-detected titration experiments. The solid line is to visually guide the eye. (D) Representative DOG-binding curves of the W252Y variant constructed based on the NMR-detected titration data. The solid lines are the global fits to Eq. 2.

FIGURE 4. Ligand-independent interactions of wtC1Bδ with micelles. (A) CSP analysis for the pair of apo and DPC-bound states. Residue-specific Δ values were calculated according to Eq. 1; the mean and (mean + standard deviation, SD) Δ values are marked with blue and red lines, respectively. “B” denotes residues that are exchange-broadened and therefore unavailable for comparison; P241 is labeled with an asterisk. Residues with Δ exceeding the mean and (mean+SD) values are mapped onto the apo C1Bδ structure in blue and red, respectively, while residues with no data and P241 are shown in black. (B) wtC1Bδ binding curves constructed by plotting the CSP value Δ against the total concentration of micelles. Solid lines represent the global fit of all DPC-responsive residues. (C) CSP analysis for the pair of apo and DPS/DPC-bound C1Bδ states. (D) Two types of binding sites observed in wtC1Bδ upon interaction with mixed micelles. Solid lines represent the global fit of all micelle-responsive residues.
within a specific group. (E) A model of wtC1Bδ interactions with mixed micelles. Events 1-3 are described in the text.

FIGURE 5. α1 residues in the ternary C1Bδ-DOG-micelle complex are influenced by the presence of PtdSer. (A) Overlay of the 15N-1H HSQC spectra collected on a 100 µM [U-15N] enriched C1Bδ in three states: apo (green), DOG-complexed in DPC-only micelles (blue), and DOG-complexed in mixed DPS/DPC micelles (red). The total concentration of C1Bδ, DOG, and detergent was 100 µM, 500 µM, and 10 mM, respectively. The displacement of cross-peaks is indicated with arrows: from the apo- to DOG/DPC-bound states (solid arrows) and from the DOG/DPC-bound to the DOG/DPS/DPC-bound states (dashed arrows). The backbone N-H resonances of W252, Q257, and G258 (labeled with asterisks) are exchange-broadened in apo C1Bδ. Residues K271, H270, and E274, whose chemical shifts are affected by DPS but not DPC, are in boldface. (B) The chemical shift perturbation Δ calculated for the pair of DOG/DPS/DPC-bound and DOG/DPC-bound C1Bδ states. The mean and (mean+SD) Δ values of 0.05 and 0.14 are marked with blue and red lines, respectively.

FIGURE 6. W252Y mutation decreases the affinity of C1Bδ to mixed micelles. (A) CSP analysis for the pair of apo and DPS/DPC-bound W252Y states. Residue-specific Δ values were calculated according to Eq. 1; the mean and (mean + standard deviation, SD) Δ values are indicated with blue and red lines, respectively. “B” denotes residues that are exchange-broadened in W252Y and therefore unavailable for comparison; P241 and spectrally unresolved Y238 are labeled with asterisks. Residues with Δ exceeding the mean and (mean+SD) values are mapped onto the apo C1Bδ structure in blue and red, respectively, while residues with no data and P241 are shown in black. (B),(C) Two types of binding sites with distinct affinities observed in W252Y upon interaction with mixed micelles. Solid lines represent the global fit of all micelle-responsive residues within a specific group. (D) Summary of apparent dissociation constants determined for both C1Bδ variants.

FIGURE 7. Residue identity at position 252 and interactions with DOG influence the depth of C1Bδ insertion into micellar environment. The intensity ratios I_{para}/I_{dia} of the N-H resonances of wtC1Bδ (A) and W252Y (B), complexed to paramagnetic and diamagnetic preparations of mixed DPS/DPC micelles, in the absence (black bars) and presence (green bars) of DOG. Residues whose broadening in the micelle-bound state is unrelated to PRE are labeled with “B”. For each protein sample, the ratios were normalized to G281, the most C-terminal residue. The total concentrations were: 150 µM protein, 100 mM total detergent, 0.5 mM DOG, and 1.3 mM 14-doxyl PC. In (B), Δ(I_{para}/I_{dia}) is the difference in I_{para}/I_{dia} ratios between the DOG-bound and micelle-only W252Y. (C) Intensity ratios of W252Y mapped onto the structure of apo C1Bδ as a color gradient. Residues for which no data are available are shown in dark grey. Residues with significantly different insertion depth in the absence and presence of DOG are in boldface and underlined. (D) Correlation of the PRE values for the backbone N-H groups of C1Bδ variants. Residues with significantly different PRE values in the wtC1Bδ and its W252Y variant are labeled. All of them with the notable exception of His270 belong to the loop hinge regions.

FIGURE 8. Kyte-Doolittle (KD) hydropathy plots generated using the ProtScale tool of the ExPaSy Bioinformatics Resource Portal for C1B domains of α, β, and δ PKC isoenzymes from *R. norvegicus*. The residue numbering corresponds to PKCδ. The primary structure of α1 (residues 270-275) is given in the inset. Positions 273-274 that give rise to the differences in hydropathy values are highlighted with grey. Residues implicated in the interactions with PtdSer are marked with asterisks.
Figure 1
Figure 2
Figure 3

[Image: Figure 3 showing data points and chemical shifts for different conditions.]
Figure 4
Figure 5

A  

B  

Diacylglycerol affinity of C1 domains
Figure 6

A. 3.33 mM [DPS/DPC] vs Apo W252Y C1Bδ

B. Kd = 134 ± 7 µM

C. Kd = 364 ± 34 µM

D. C1Bδ•DOG•Micelle + W252Y•DOG•Micelle → C1Bδ•Micelle + W252Y•Micelle

V252, L253, and G255 are shown in red.
Figure 7
Figure 8
Interfacial Partitioning of a Loop Hinge Residue Contributes to Diacylglycerol Affinity of Conserved Region 1 Domains
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