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Probing the Determinants of Diacylglycerol Binding Affinity in the C1B Domain of Protein Kinase Cα

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C1 domains are independently folded modules that are responsible for targeting their parent proteins to lipid membranes containing diacylglycerol (DAG), a ubiquitous second messenger. The DAG binding affinities of C1 domains determine the threshold concentration of DAG required for the propagation of signaling response and the selectivity of this response among DAG receptors in the cell. The structural information currently available for C1 domains offers little insight into the molecular basis of their differential DAG binding affinities. In this work, we characterized the C1B domain of protein kinase Cα (C1Bα) and its diagnostic mutant, Y123W, using solution NMR methods and molecular dynamics simulations. The mutation did not perturb the C1Bα structure or the sub-nanosecond dynamics of the protein backbone, but resulted in a >100-fold increase in DAG binding affinity and a substantial change in microsecond timescale conformational dynamics, as quantified by NMR rotating-frame relaxation-dispersion methods. The differences in the conformational exchange behavior between wild type and Y123W C1Bα were localized to the hinge regions of ligand-binding loops. Molecular dynamics simulations provided insight into the identity of the exchanging conformers and revealed the significance of a particular residue (Gln128) in modulating the geometry of the ligand-binding site. Taken together with the results of binding studies, our findings suggest that the conformational dynamics and preferential partitioning of the tryptophan side chain into the water-lipid interface are important factors that modulate the DAG binding properties of the C1 domains.

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Abbreviations used: DAG, diacylglycerol; C1Bα, C1B domain of protein kinase Cα; PE, phorbol ester; PKC, protein kinase C; PtdSer, phosphatidylserine; wt, wild type; MD, molecular dynamics; HSQC, heteronuclear single-quantum coherence; DPC, [$^2H_{38}$] dodecylphosphocholine; DPS, 2-dihexanoyl-*sn*-glycero-3-[phospho-L-serine]; DOG, 1,2-dioctanoyl-*sn*-glycerol; RDC, residual dipolar coupling; PDBu, phorbol-12,13-dibutyrate; nOe, nuclear Overhauser enhancement; CPMG, Carr-Purcell-Meiboom-Gill.

Introduction

"Typical" C1 domains[1] are independently folded modules of ~50 amino acids that regulate the function of at least seven families of signaling proteins. These proteins, collectively referred to as diacylglycerol (DAG)/phorbol ester (PE) receptors, include novel and conventional protein kinase C (PKC) isoenzymes, protein kinase D, chimerins, RasGRPs, Unc-13/Munc-13 proteins, DAG kinases, and myotonic-dystrophy-kinase-related Cdc42-binding kinases (reviewed by Brose and Rosenmund,[2] Kazanietz,[3]

and Blumberg *et al.*[4]). The function of typical C1 domains within their parent proteins is to associate with lipid membranes in response to binding DAG (their natural agonist) or PEs. PEs are naturally occurring tetracyclic diterpenoids with known tumor-promoter activity.[5] They have found widespread application as pharmacological and research tools for studying the function of PKCs and their role in carcinogenesis.[6,7]

PKC was identified as the first DAG/PE receptor by Nishizuka's laboratory in 1982.[8] In conventional and novel PKCs, two C1 domains are found as a tandem and are designated C1A and C1B.[9,10] The membrane-binding event mediated by C1 domains releases the autoinhibitory interaction between the N-terminal pseudo-substrate region and the kinase active site,[11] thereby activating the enzyme. In conventional PKCs comprising α, βI, βII, and γ isoforms, the membrane recruitment step also involves the C2 domain,[12,13] which undergoes membrane insertion in response to binding Ca^{2+} and phosphatidylserine (PtdSer). Both C1 and C2 domains preserve their ligand binding properties in their respective isolated forms.

Despite their high sequence identity, C1A and C1B domains within the same PKC isoenzyme have different intrinsic affinities for DAG and PEs, with the notable exception of PKCγ. This was demonstrated for both isolated domains[14,15] and full-length PKC.[16] Distinct DAG and PE affinities of C1 domains directly translate into their individual functional roles within the parent PKC isoenzyme. In a series of elegant experiments, Medkova and Cho showed that mutations of essential hydrophobic residues in the C1A domain, but not in the C1B domain, dramatically reduced the binding of full-length PKCα to DAG-containing vesicles, its enzymatic activity, and the depth of monolayer penetration.[17] A similar pattern was observed for PKCδ, where C1A and C1B domains were shown to have nonequivalent roles in mediating the kinase activation response to DAG[18] and PE.[19] Taken together, these findings indicate that the C1A and C1B domains are responsible for the DAG- and PE-induced activation of PKCs, respectively. Ligand preferences of C1 domains also play a major role in determining the subcellular localization of PKC activity and may control the isoform-specific response in the case of overlapping PKC functions. In addition, C1 domains have been identified as targets for designing therapeutic agents that selectively inhibit or activate PKCs.[3,4] Despite the significance of C1 domains as regulatory modules, the determinants of their ligand specificities and binding affinities are poorly understood.

The available structural information on C1 domains reveals little about the molecular basis of their ligand preferences. All domains share the basic elements that include two structural Zn^{2+},[20] a hydrophobic ridge surrounding the DAG-binding site, and a belt of basic residues across the middle part of the protein. A comparison of primary structures for several C1 domains is shown in Fig. 1. The residues involved in Zn^{2+} coordination are highlighted in blue and include three cysteines and one histidine per Zn^{2+} site. The ligand (DAG or PE) binds between loop β12 and loop β34. In addition to Zn^{2+}-binding residues, Pro112, Gly124, and the Gln128-Gly129 motif constitute the consensus sequence for the typical C1 domains.

A search of the Protein Data Bank yielded 14 unique structures of isolated C1 domains, of which one was determined by X-ray crystallography and

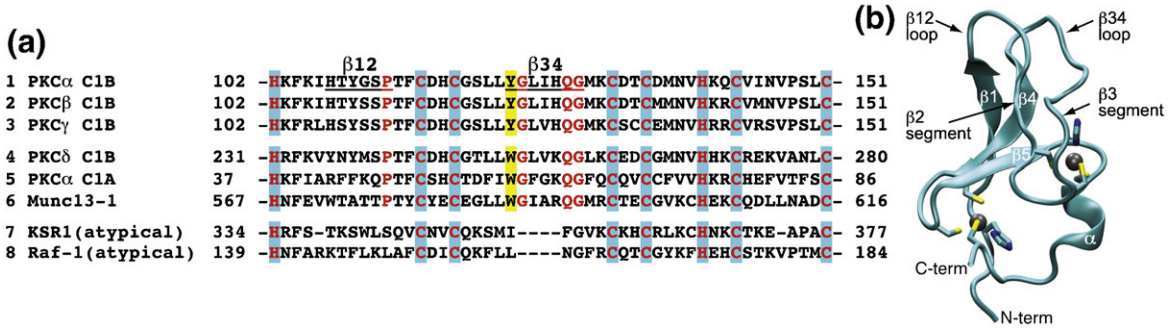

Fig. 1. (a) Comparison of the primary structures of DAG/PE-responsive (1 through 6) and "atypical" (7 and 8) C1 domains. C1Bα numbering is used to indicate the amino acid position in the primary structure. The consensus sequence for DAG/PE-responsive C1 domains (red) comprises residues involved in coordinating two structural Zn^{2+} (highlighted in blue), Pro112, Gly124, and the Gln128-Gly129 motif. DAG-binding loops β12 and β34 are underlined in C1Bα. Loop β34 in atypical C1 domains from KSR-1 and Raf-1 has a 4-amino-acid deletion.[21,22] A conserved Tyr at position 123 is highlighted in yellow. All C1 sequences are from *Mus musculus*, except for Raf1, which is from *Homo sapiens*. (b) Ribbon representation of the ensemble-averaged NMR structure of C1Bα.[23] The coordinates were kindly provided by Dr. Ulrich Hommel β2 and β3 segments are identified as 3-amino acid β-strands by Hommel *et al.* and will be referred to as such in the remainder of the article.[23]