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Diacylglycerol kinase γ is a novel anionic phospholipid binding protein with a selective binding preference

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ABSTRACT

There are ten isozymes of diacylglycerol kinase (DGK), and they regulate diverse patho-physiological functions. Here, we investigated the lipid-binding properties of DGK isozymes using protein–lipid overlay and liposome-binding assays. DGKγ showed a strong binding activity compared with other DGK isozymes for phosphatidic acid (PA) among the various glycerophospholipids tested. However, DGKγ failed to interact with DG and lyso-PA. Moreover, the isozyme was capable of binding to ceramide-1-phosphate but not to ceramide or sphingosine-1-phosphate. The isozyme bound more strongly to PA containing unsaturated fatty acid than to PA having only saturated fatty acid. An analysis using a series of deletion mutants of DGKγ revealed that the N-terminal region, which contains a recoverin homology domain and EF-hand motifs, is responsible for the PA binding activity of DGKγ. Taken together, these results indicate that DGKγ is an anionic phospholipid binding protein that preferably interacts with a small highly charged head group that is very close to the glycerol or sphingosine backbone.

1. Introduction

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) to convert it to phosphatidic acid (PA) [1–5]. DG is known to be an activator of the conventional and novel protein kinase Cs, Ras guanyl nucleotide-releasing protein, Unc-13 and chimaerin [6–8]. PA also controls a variety of important signaling proteins, such as phosphatidylinositol-4-phosphate 5-kinase, son of sevenless, Ras GTPase-activating protein, C-Raf and atypical protein kinase C [7,9]. Therefore, DGK appears to participate in various physiological events by modulating the balance between two bioactive lipids, DG and PA, in microenvironments within cells.

Ten mammalian DGK isozymes (α, β, γ, δ, ε, ζ, η, θ, ι and κ) that share two or three characteristic zinc finger-like C1 domains and the catalytic region of the enzyme in common, are divided into five groups according to their structural features [1–5]. For example, the type I DGK isozymes, DGKs α, β and γ [10,11], commonly contain two consecutive EF-hand motifs, so they are also members of the EF-hand family of Ca^{2+}-binding proteins. In addition to two sets of Ca^{2+}-binding EF hand motifs, all type I DGK isozymes possess the same domain structures: an N-terminal recoverin homology (RVH) domain, two cysteine-rich C1 domains and the C-terminal catalytic domain. The type II DGK isoforms, DGKs δ, η and κ, possess a pleckstrin homology domain at their N-termini and a separate catalytic region and a sterile α-motif domain at their C-termini. The type III DGK isoform, DGKε, has no recognizable functional domains except for the C1 domains and the catalytic region. The type IV isozymes, DGKs ζ and ι, are characterized by a myristoylated alanine-rich C kinase substrate phosphorylation site domain and four ankyrin repeats. A type V isoform, DGKθ, has three (instead of two) C1 domains, a Gly/Pro-rich domain and a pleckstrin homology domain-like region with an overlapping Ras-associating domain. Recent studies have revealed that DGK isozymes play important roles in a wide variety of signal transduction events involved in development, neural networking, immune responses, cytoskeletal reorganization, glucose incorporation and carcinogenesis [1–5].

Several DGK isozymes were reported to be localized and translocated to the plasma membrane and intracellular membranes such as endosomes [12–14]. Moreover, many DGK isozymes are known to be activated by phosphatidylserine (PS) [15,16]. These results imply that membrane lipids and DGK isozymes are potentially related. However, the lipid-binding properties of DGK

Abbreviations: Cer, ceramide; C1P, ceramide 1-phosphate; CL, cardiolipin; DG, diacylglycerol; DGK, diacylglycerol kinase; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; RVH, recoverin homology; SM, sphingomyelin; WT, wild type.

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isozymes are poorly understood. To characterize their lipid-binding properties, we comprehensively analyzed the lipid-binding abilities of ten DGK isozymes using a protein–lipid overlay assay.

2. Materials and methods

2.1. Plasmid constructs

The expression plasmids p3×FLAG-CMV-pig DGKα [17], -rat DGKβ [17], -human DGKγ [17], -human DGKδ [18,19] -human DGKε [20], -human ζ [20], -human DGKη [21], -human ι [20], -human θ [20] and -human DGKκ [22] were generated as described. The cDNA encoding DGKγ-1–259, which contains the recoverin homology domain and two EF-hand motifs (amino acids 1–259), and the cDNA encoding DGKγ-260–791, which contains two C1 domains and a catalytic domain, were generated from a human DGKγ cDNA clone (amino acids 260–791) [23] by PCR and subcloned into the p3×FLAG-CMV-7.1 (Sigma–Aldrich) and pAcGFP-C1 (Takara-Clontech) vectors [23].

2.2. Cell culture and cDNA transfection

Cell culture was performed as described previously [20]. cDNAs were transfected into COS-7 cells by electroporation with the Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories) according to the manufacturer's instructions.

2.3. Western blot analysis

Western blot analysis was carried out as described previously [20].

2.4. Protein–lipid overlay assay

100 pmol of the indicated lipids were spotted onto a nitrocellulose membrane (Bio-Rad Laboratories). The membranes were subjected to blocking with 1% skim milk/Tris-buffered saline, pH 7.2, for 1 h at room temperature. After the blocking, 10 ml of 3% fatty acid-free bovine serum albumin/Tris-buffered saline, pH 7.2, in which 200 μl of the cell lysates containing the protein of interest were diluted, was added to the membranes. The membranes were then incubated for 30 min at room temperature and then at 4 °C overnight. The membranes were incubated with the anti-FLAG M2 antibody, anti-c-myc antibody or anti-DGKγ antiserum [17] followed by incubation with the peroxidase-conjugated anti-mouse IgG antibody or goat anti-rabbit IgG antibody. Finally, the lipid bound proteins were visualized using the ECL Western-blotting detection system. Quantitative densitometry was performed using the Image J software.

3. Results

3.1. PA- and PS-binding activities of DGK isozymes

To detect the physical interactions between the DGK isozymes (α–κ) and lipids, binding studies were carried out using the lipid–protein overlay method (fat-blot assays) as described previously [24,25]. Because many DGK isozymes were reported to be activated by PS [15,16], an acidic phospholipid, we first performed a protein–lipid overlay assay of ten DGK isozymes with PS. The binding abilities of the ten DGK isozymes with PA, another acidic phospholipid, were also analyzed. PS and PA were immobilized on a nitrocellulose membrane, and binding was examined by incubating the membrane with cell lysates from COS-7 cells overexpressing FLAG-tagged DGK isozyme proteins followed by immunostaining with an anti-FLAG antibody. The expression levels of these isozymes were found to be comparable to each other by Western blotting with an anti-FLAG antibody (Fig. 1A). Because no significant band was detected in the lysates from the cells transfected with vector alone, several minor crossreactive bands with lower molecular masses would be truncated products of the isozymes. Although PS is a DGK activator, it showed only weak or no detectable binding activity against all of the DGK isozymes tested. Interestingly, DGKγ had a strong binding affinity for PA (Fig. 1B and C). On the other hand, the PA-binding abilities of the other DGK isozymes were markedly lower than that of DGKγ (Fig. 1B and C).

To deny the possibility that the FLAG tag might affect the PA-binding activity of DGKγ, we next examined whether PA showed strong binding activity when subjected to an overlay assay using DGKγ with a different tag or without a tag (no tag). The expression of myc-tagged DGKγ and the protein without a tag was confirmed by Western blotting (Suppl. Fig. 1A and C). As shown in Suppl. Fig. 1B, myc-tagged DGKγ clearly and intensely associated with PA. Moreover, the protein without a tag also showed a robust PA-binding ability (Suppl. Fig. 1D). These results indicate that the protein tag has no or very little effect on the binding ability of DGKγ to PA.

To independently verify PA binding, a liposome-binding assay was performed. In this assay, liposomes of defined lipid composition

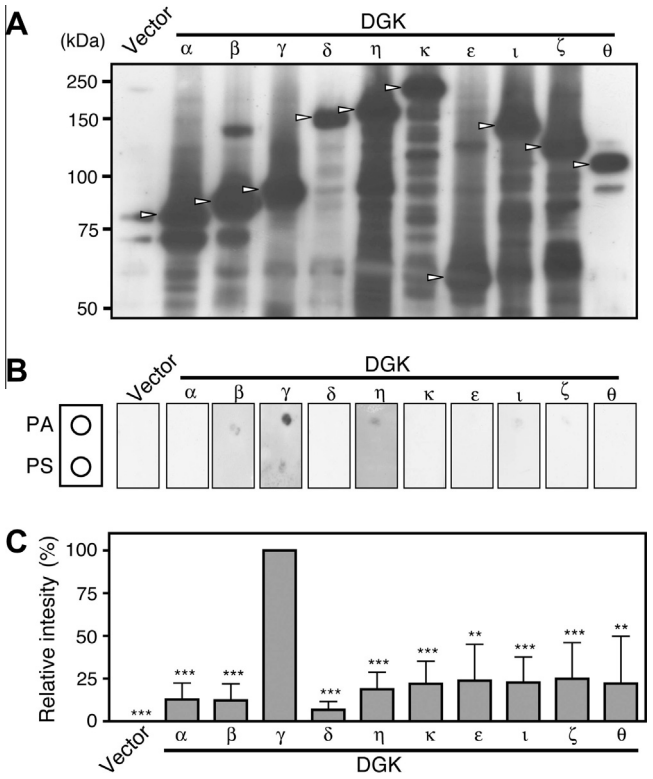

Fig. 1. Comparison of binding activities of DGK isozymes to phosphatidic acid (PA) and phosphatidylserine (PS). (A) 3×FLAG tagged DGK isozymes expressed in COS-7 cells were detected by Western blotting with anti-FLAG antibody. Results are representative of three independent experiments. (B) Equimolar amounts (100 pmol) of 18:1/18:1-PA and PS were immobilized on nitrocellulose membranes and probed with 3×FLAG-tagged DGK isozyme-expressing or non-expressing COS-7 cell lysates. The lipid–protein binding was identified by immunostaining with anti-FLAG antibody. Results are representative of three independent experiments. (C) The blots were scanned and quantified using Image J software. PA-binding levels of DGKγ were set to 100%. The data are shown as the means ± SD of three independent experiments. Statistical significance compared with DGKγ was determined using Student's *t* test (***P* < 0.01, ****P* < 0.005).

were incubated with DGKγ. Following centrifugation, protein bound to the liposomes was detected in the pellet while unbound protein remained in the supernatant. Because a protein–lipid overlay assay demonstrated that phosphatidylcholine (PC) did not show detectable binding activity to DGKγ (see Fig. 2), we prepared two types of liposomes containing PC alone and both PC and PA (9:1 ratio). Using this assay, DGKγ was found to strongly bind to the PA-containing liposomes, but not to the liposomes containing only PC (Suppl. Fig. 2). On the other hand, DGKα did not show a marked affinity for the PA-containing liposomes (Suppl. Fig. 2).

3.2. Lipid binding properties of DGKγ

To examine the lipid-binding properties of DGKγ we investigated its binding to various phospholipids. 100 pmol of PA and other phospholipids (PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), cardiolipin (CL), phosphatidylglycerol (PG) and PS), and various amounts of PA, PC, CL and PS were spotted onto nitrocellulose membranes as shown in Fig. 2A and C, and were subjected to overlay assays with DGKγ. We found that of all the phospholipids tested, PA bound most strongly to DGKγ (Fig. 2A, C and D). PS showed only weak binding activity to DGKγ (Fig. 2A, C and D), and PC, PE, PI and PG failed to show marked binding activities to DGKγ (Fig. 2A and D). CL showed relatively strong binding activity to DGKγ (Fig. 2A, C and D); however, it should be noted that CL is predominantly localized to the inner membrane of the mitochondrion. Because DGKγ is localized to the cytoplasm and plasma membrane, we chose to focus the subsequent studies on PA. Other isozymes (α, β, δ, ε, ζ, η, ι, θ and κ) showed significantly low or no detectable binding activities against PC, PE, PI, CL and PG (data not shown).

The sphingolipids SM and Sph were spotted and were subjected to an overlay assay with DGKγ (Fig. 2A); however, DGKγ did not markedly bind to these lipids (Fig. 2A and D).

We next investigated the binding activities of DGKγ to two PA-related lipids, DG and lysoPA (LPA). No detectable binding activities of DGKγ to DG or LPA were detected (Fig. 2B and D). The binding activities to Cer-1-phosphate (C1P) and its related lipids, Cer and sphingosine-1-phosphate (S1P), were also determined. Interestingly, DGKγ strongly associated with C1P, whereas Cer and S1P failed to bind to the protein (Fig. 2B–D). Taken together, among various glycero- and sphingo-lipids, DGKγ intensely binds to PA and, to a lesser extent, C1P.

To further assess the DGKγ binding properties to PA, DGKγ was subjected to an overlay assay with PA species with different types of fatty acid chains, including 18:1/18:1-PA, 18:0/20:4-PA, 14:0/14:0-PA, 18:0/18:0-PA and chicken egg yolk PA, which mainly contains 18:1/18:1-PA. Fig. 3 showed that DGKγ more strongly bound to the PA species that contained at least one unsaturated fatty acid, 18:0/20:4-PA, 18:1/18:1-PA and chicken egg yolk PA, than to the PA species with only saturated fatty acids, 18:0/18:0-PA and 14:0/14:0-PA. Therefore, it was suggested that DGKγ has a preference for unsaturated fatty acid-containing PA species.

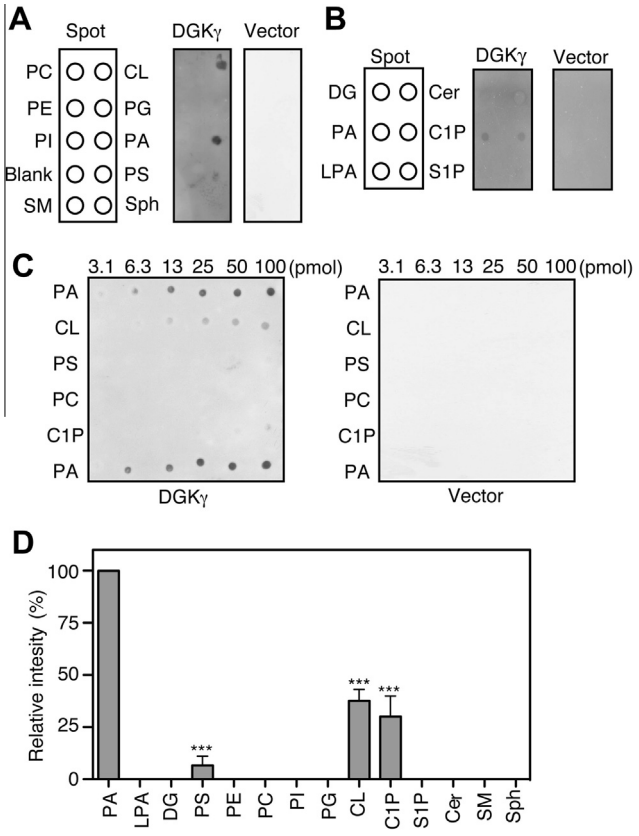

Fig. 2. DGKγ selectively binds to PA, CL and C1P. (A) Equimolar amounts (100 pmol) of PC, PE, PI, SM, CL, PG, 18:1/18:1-PA, PS and Sph were spotted onto nitrocellulose membranes as indicated. The membranes were incubated with 3×FLAG-tagged DGKγ-expressing or non-expressing COS-7 cell lysates. Lipid-bound DGKγ was detected by immunostaining with anti-FLAG antibody. (B) Equimolar amounts (100 pmol) of DG, 18:1/18:1-PA, LPA, Cer, C1P and S1P were blotted onto nitrocellulose membranes, and membranes were incubated with 3×FLAG-tagged DGKγ-expressing COS-7 cell lysates. Lipid-bound DGKγ was detected with anti-FLAG antibody. (C) Indicated amounts of 18:1/18:1-PA, CL, PS, PC and C1P were spotted onto a nitrocellulose membrane. The membrane was incubated with 3×FLAG-tagged DGKγ-expressing or non-expressing COS-7 cell lysates. Lipid-bound DGKγ was detected by immunostaining with anti-FLAG antibody. (D) The blots (100 pmol lipids) were scanned and quantified using Image J software. PA-binding levels of DGKγ were set to 100%. The data are shown as the means ± SD of three independent experiments. Statistical significance compared with PA was determined using Student's *t* test (***$P < 0.005$).

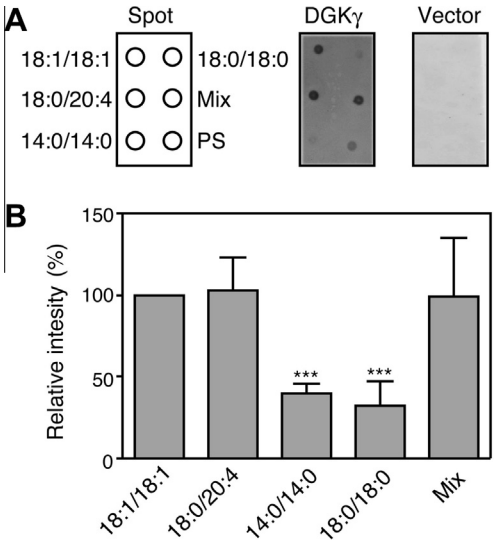

Fig. 3. Binding of DGKγ to PA species with various fatty acid chains. (A) Equimolar amounts (100 pmol) of 18:1/18:1-PA, 18:0/20:4-PA, 14:0/14:0-PA, 18:0/18:0-PA and PA derived from chicken egg yolk (Mix) were spotted onto nitrocellulose membranes as indicated. The membranes were incubated with 3×FLAG-tagged DGKγ expressing or non-expressing COS-7 cell lysates. Lipid-bound DGKγ was detected by immunostaining with anti-FLAG antibody. (B) The blots were scanned and quantified using Image J software. 18:1/18:1-PA-binding levels of DGKγ were set to 100%. The data are shown as the means ± SD of three independent experiments. Statistical significance compared with 18:1/18:1-PA was determined using Student's *t* test (*$P < 0.05$).

3.3. PA-binding region of DGKγ

We next attempted to identify which part in DGKγ mainly contributes to its PA binding. To this end, two 3×FLAG-tagged deletion mutants, DGKγ-1–259 containing RVH domain and two EF-hand motifs and DGKγ-260–791 containing two C1 domains and a catalytic domain (Fig. 4A), were subjected to an overlay assay. It was confirmed that the expression levels of wild type (WT) DGKγ, DGKγ-1–259 and DGKγ-260–791 were comparable to each other (Fig. 4B). As shown in Fig. 4C and D, DGKγ-1–259 showed strong binding to PA, whereas DGKγ-260–791 exhibited very weak PA-binding activity. The results indicated that the N-terminal region having the recoverin homology domain and two EF-hand motifs is responsible for the PA-association ability of DGKγ.

4. Discussion

The lipid-binding properties of DGK isozymes are poorly understood currently. To shed light on this issue, we comprehensively analyzed the lipid-binding activities of ten DGK isozymes using a protein–lipid overlay assay. In this study, we found that, among ten DGK isozymes, DGKγ alone has an intense PA-binding activity (Figs. 1 and 2). Therefore, DGKγ is currently recognized as a new PA-binding protein. In addition, this isozyme bound to C1P (Fig. 2). No protein that binds both PA and C1P has been reported to date. Thus, it appears that DGKγ is a new type of anionic phospholipid binding protein.

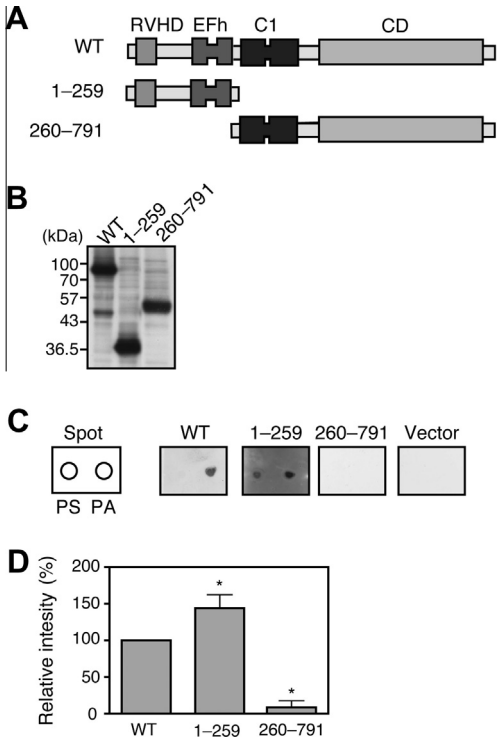

Fig. 4. PA-binding of wild-type (WT) DGKγ and its mutants. (A) Schematic representation of DGKγ mutants used (RVHD, Recoverin homology domain; EF-h, EF-hand motif; C1, C1 domain; CD, catalytic domain). (B) 3×FLAG-tagged DGKγ, DGKγ-1–259 and DGKγ-260–791 were expressed in COS-7 cells. The Western blot analysis was performed with anti-FLAG antibody as described in "Section 2". (C) Equimolar amounts (100 pmol) of 18:1/18:1-PA and PS were spotted onto nitrocellulose membranes as indicated. The membranes were incubated with COS-7 cell lysates containing 3×FLAG-tagged DGKγ (WT) or its deletion mutants. (D) The blots were scanned and quantified using Image J software. The 18:1/18:1-PA-binding levels of DGKγ (WT) were set to 100%. The data are shown as the means ± SD of three independent experiments. Statistical significance compared with WT DGKγ was determined using Student's *t* test (*$P < 0.05$).

The lipid–protein overlay assays of other PA-binding motifs reported so far were performed with PA of 5–50 nmol (protein phosphatase-1) [26], 6.3–25 nmol (neutral sphingomyelinase 2) [27] and 1–10 nmol (trigalactosyldiacylglycerol 4 protein) [28,29]. On the other hand, in this study, we spotted only 3.1–100 pmol of PA (Figs. 1–4 and Suppl. Fig. 1). The results allow us to speculate that the PA-binding activity of DGKγ is stronger than those of the PA-binding proteins studied previously, and that the PA-DGKγ binding occurs under more physiological conditions.

Among various phospholipids, PA and C1P showed the strongest binding activities for DGKγ (Fig. 2); however, DG, LPA, Cer and S1P failed to associate with that isozyme (Fig. 2). Thus, the DGKγ association with the lipids structurally requires that the ligand has both a phosphate head group and two acyl chains. Moreover, PA and C1P have a common feature in their structures. PA is a unique acidic phospholipid because it has a small highly anionic charged head group that is very close to the glycerol backbone. C1P also has a small highly anionic charged head group that is very close to the sphingosine backbone. It is thus possible that the PA-binding region of DGKγ recognizes the common structural feature of PA and C1P.

DGKγ showed stronger binding to PA species possessing at least one unsaturated fatty acid, 18:0/20:4-PA, 18:1/18:1-PA and chicken egg yolk PA, compared with PA species having only saturated fatty acids, 18:0/18:0-PA and 14:0/14:0-PA (Fig. 3). These results indicate that DGKγ prefers unsaturated fatty acid-containing PA species to those containing only saturated fatty acids.

The RVH and EF-hand motif domains of DGKγ (DGKγ-1–259) expressed in and purified from *Escherichia coli* cells did not show significant PA-binding activity (data not shown). This result suggests that post-transcriptional modification is required for the PA-binding of DGKγ. Alternatively, unknown DGKγ-specific factor(s) in mammalian cells may affect the PA-binding activity of DGKγ. It will be interesting to determine what causes this discrepancy between the bacterially expressed DGKγ and the protein expressed in mammalian cells.

The N-terminal region of DGKγ, which contains the RVH domain and two EF-hand motifs, was implicated as the PA-binding region of the isozyme (Fig. 4). To identify which domain/motif in the N-terminal region (DGKγ-1–259) contributes to the PA binding, we generated 3×FLAG-tagged constructs, DGKγ-1–173 containing the recoverin homology domain and DGKγ-174–256 with only the EF-hand motifs. However, the expression level of DGKγ-174–256 was extremely lower than those of DGKγ-1–173 and DGKγ-1–259 (data not shown). Therefore, we could not further narrow down the PA-binding region of this isozyme.

DGKα and DGKβ, which are type I DGK subfamily members in addition to DGKγ, also possess the RVH domain and EF-hand motifs at their N-terminus. However, these isozymes did not strongly bind to PA (Fig. 1). DGKγ has 63 aa and 33 aa extensions compared with DGKα and DGKβ, respectively. The differences in the amino acid sequences between DGKγ and DGKα/β may result in their differing affinities for PA.

Many PA-binding motifs were so far reported as follows: "FPCYWXXDRLXASXXXTXXEKRXR" and "LXLLXXXLPFAXXGFXXWXP XQXXR(R/K)" in neutral sphingomyelinase 2 [27], "QXXFFXRFMXSP XXPXFSXXR" in the *Arabidopsis* trigalactosyldiacylglycerol 4 protein [29], DETLMCSFQIL in the catalytic subunit (γ isoform) of protein phosphatase-1 [30], "LVXWWDXXKR" in the unique N-terminal region of the cyclic AMP-specific phosphodiesterase PDE4A1 [31], "RKTRXXILLFM" in Raf-1 (C-Raf) [32], "NHXXPRLP" in the PH domain of Sos [33] and "RRVGRPRSM–RSKKRT" in DOCK2 [34]. However, no similar sequences were found in the RVH domain and EF-hand motifs-containing N-terminal region of DGKγ.

Recent studies have begun to uncover the biological functions of DGKγ. We previously reported that DGKγ negatively regulated

macrophage differentiation in human leukemia HL-60 and U937 cells through its catalytic action [35]. We also demonstrated that the γ-isozyme, through its catalytic action, served as a novel upstream suppressor of Rac1 and consequently, as an attenuator of lamellipodium/ruffle formation in NIH3T3 fibroblasts [17]. Moreover, DGKγ interacted with and activates β2-chimaerin, a Rac-specific GAP, in response to epidermal growth factor [36]. DGKγ induced filopodia-like protrusions in N1E-115 neuroblastoma cells independent of its enzymatic activity [23]. Future studies exploring the regulatory function of PA and the mechanism by which PA affects DGKγ function will provide further insights into the physiological roles of this enzyme.

In summary, our data indicate that DGKγ is an anionic phospholipid binding protein that preferably interacts with a small highly charged head group that is very close to the glycerol or sphingosine backbone such as PA and C1P.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.116.

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