FEBS Open Bio 4 (2014) 387–392

FEBS openbio

journal homepage: www.elsevier.com/locate/febsopenbio

EF-hand motifs of diacylglycerol kinase α interact intra-molecularly with its C1 domains

Tatsuya Yamamoto, Hiromichi Sakai, Fumio Sakane *

Department of Chemistry, Graduate School of Science, Chiba University, Chiba, Japan

ARTICLE INFO

Article history:
Received 3 April 2014
Revised 14 April 2014
Accepted 15 April 2014

Keywords:
Diacylglycerol kinase
Calcium
C1 domain
EF-hand
Intra-molecular interaction

ABSTRACT

Diacylglycerol kinase (DGK) α, which is activated by Ca^{2+}, contains a recoverin homology (RVH) domain, tandem repeats of two Ca^{2+}-binding EF-hand motifs, two cysteine-rich C1 domains and the catalytic domain. We previously found that a DGKα mutant lacking the RVH domain and EF-hands was constitutively active and that the N-terminal region of DGKα, consisting of the RVH domain and EF-hand motifs, interacted intra-molecularly with the C-terminal region containing the C1 and catalytic domains. In this study, we narrowed down the interaction regions of DGKα. At the C-terminal region, the C1 domains are responsible for the intra-molecular interaction. At the N-terminal region, the EF-hand motifs mainly contribute to the interaction. Moreover, using highly purified EF-hand motifs and C1 domains, we demonstrate that they directly bind to each other. The co-precipitation of these two domains was clearly attenuated by the addition of Ca^{2+}. These results indicate that the Ca^{2+}-induced dissociation of the intra-molecular interaction between the EF-hand motifs and the C1 domains of DGKα is the key event that regulates the activity of the enzyme.

1. Introduction

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) to generate phosphatidic acid (PA) [1–5]. DG is an established activator of the conventional and novel protein kinase Cs, Ras guanyl nucleotide-releasing protein, Unc-13 and chimaerin [6,7]. PA also regulates various important signaling factors, such as phosphatidylinositol-4-phosphate 5-kinase, son of sevenless, Ras GTPase-activating protein, C-Raf and atypical protein kinase C [3,8–10]. Therefore, DGK is thought to function in a variety of physiological events by modulating the balance between two signaling lipids, DG and PA.

Ten mammalian DGK isozymes (α, β, γ, δ, ε, ζ, η, θ, ι and κ), which share two or three characteristic zinc finger-like C1 domains and the catalytic region of the enzyme, are divided into five groups according to their structural features [1–5]. Type I DGK isozymes (DGKs α, β and γ) commonly contain tandem repeats of two EF-hand motif domains and are classified as members of the EF-hand family of Ca^{2+}-binding proteins. In addition to the Ca^{2+}-binding EF hand motifs, all type I DGK isozymes contain an N-terminal recoverin homology (RVH) domain, two cysteine-rich C1 domains and the C-terminal catalytic region [1–5]. Interestingly, these isozymes exhibit different tissue- and cell-specific modes of expression. DGKα is most abundant in T-lymphocytes and the thymus [11,12], oligodendrocytes of the brain [13] and melanoma cells [14]. DGKα is involved in a wide variety of pathophysiological events [15], such as interleukin-2-dependent T-cell proliferation [16], T-cell anergy [17,18], hepatocyte growth factor-induced cell motility [19], melanoma apoptosis [14] and the progression of human hepatocellular carcinoma [20].

Calcium-mediated cellular signal transduction plays an important role in the control of the physiological functions in various types of cells [21,22]. Most of the EF-hand proteins, such as calmodulin (CaM), troponin C and calcineurin regulation subunit B, are relatively small molecules (10–20 kDa) [22] that play a specialized role as Ca^{2+}-sensitive regulators of many target proteins, and their amino acid sequences are primarily composed of EF-hand motifs. In contrast, type I DGKs are relatively large for EF-hand proteins (80–90 kDa), and represent a fusion protein connected to EF-hand motifs (approximately 110 kDa) that are combined with

Abbreviations: C1Ds, C1 domains; CR, catalytic region; DG, diacylglycerol; DGK, diacylglycerol kinase; EFHs, EF-hand motifs; EGFP, enhanced green fluorescence protein; EGTA, ethylene glycol tetraacetic acid; GST, glutathione *S*-transferase; RVH, recoverin homology; RVHD, RVH domain; TF, trigger factor

* Corresponding author. Address: Department of Chemistry, Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan. Tel./fax: +81 43 290 3695.

E-mail address: sakane@faculty.chiba-u.jp (F. Sakane).

http://dx.doi.org/10.1016/j.fob.2014.04.003
2211-5463/

other functional domains, including the RVH, C1 and catalytic domains [1–5]. Calcium-activated neutral protease, calpain [23,24] and inositol phospholipid-specific phospholipase C [25] are the only known examples of this type of fusion protein with a proven capacity for Ca^{2+}.

Previously, we demonstrated that purified DGKα binds Ca^{2+} in 2:1 metal:enzyme stoichiometry with an apparent dissociation constant, K_d, of 300 nM [26,27]. The addition of Ca^{2+} in the presence of phosphatidylserine significantly activated the enzyme [26]. On the other hand, although DGK β and γ possess EF-hand motifs, the activities of these isozymes were not significantly affected by Ca^{2+} [27]. Intriguingly, a DGKα mutant lacking the RVH and the EF-hand motif domains translocated from the cytosol to the membranes [28,29] and became constitutively active [28–30]. DGKα was eluted at the monomer position (80 kDa) by gel filtration [31]. These results [28–31] imply the presence of an intra-molecular, not inter-molecular, interaction between the N-terminal region of DGKα, consisting of the RVH domain and EF hand motifs, and the C-terminal region, consisting of the C1 and catalytic domains, which masks its activity. Moreover, by expressing the N-terminal and C-terminal regions of DGKα separately, we recently showed that the two domains interact with each other and that this association is attenuated by the addition of Ca^{2+} [32]. Furthermore, we demonstrated that Ca^{2+}-induced conformational changes in the DGKα-N-terminal region disrupted the intra-molecular association between the two regions of the enzyme [32]. However, the precise domains that mediate this binding are unknown.

In this study, we attempted to narrow down the interaction domains in both the N-terminal and C-terminal regions of DGKα, and we found an intra-molecular and direct interaction between the C1 domains of DGKα and its EF-hand motifs.

2. Results

2.1. The C1 domains of DGKα interact with the N-terminal region of the enzyme

We first attempted to narrow down the region of interaction in the C-terminal region of DGKα, which contains the C1 domains and catalytic region. To this end, the C1 domains and the catalytic region of DGKα that were separately fused with enhanced green fluorescence protein (EGFP) were constructed (Fig. 1). They were then expressed in mammalian COS-7 cells in addition to EGFP connected to the C-terminal region of DGKα (85 K), and proteins with the expected molecular weights, 46 K and 68 K, respectively, were obtained (Fig. S1). Glutathione *S*-transferase (GST, 26 K), and GST-fused with the N-terminal region of DGKα (48 K) (Fig. 1; Fig. S2) were produced by bacteria expression, followed by affinity purification using glutathione–Sepharose beads.

We examined the physical interaction between the N-terminal region of DGKα and the C1 domain or catalytic region of the enzyme by conducting a co-precipitation analysis using the purified GST-fused DGKα-N-terminal region and lysates from COS-7 cells expressing the EGFP-DGKα-C1 domains or EGFP-DGKα-catalytic region. As previously reported [32], it was confirmed that the GST-DGKα-N-terminal region was co-precipitated with the EGFP-DGKα-C-terminal region (Fig. 2). As shown in Fig. 2, the C1 domains of DGKα were strongly co-precipitated with the DGKα-N-terminal region, while the catalytic region was not able to bind to the DGKα-N-terminal region. However, GST alone did not pull-down the EGFP-DGKα-C1 domains (Fig. 2). These results indicate that the C1 domains of DGKα are responsible for interacting with the N-terminal region of the enzyme.

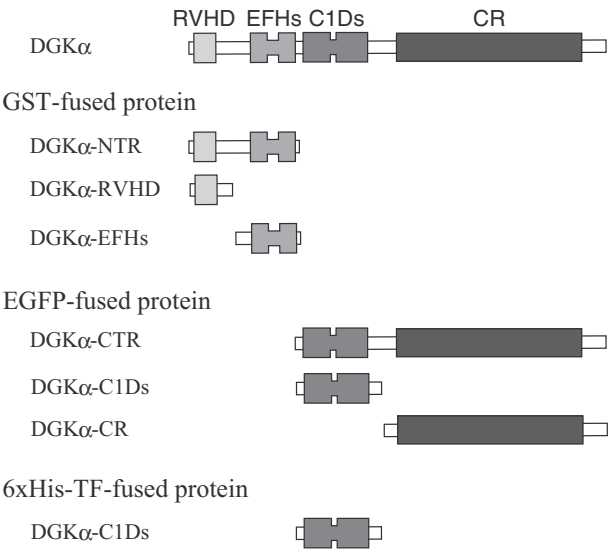

Fig. 1. Schematic representation of the DGKα mutants used in this study. *RVHD*, recoverin homology domain; *EFHs*, EF-hand motifs; *C1Ds*, C1 domains; *CR*, catalytic region; *NTR*, N-terminal region; *CTR*, C-terminal region.

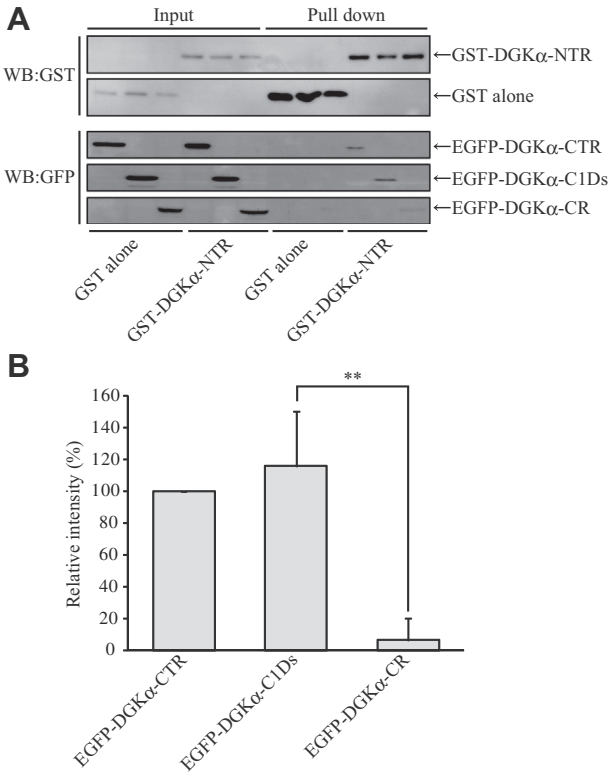

Fig. 2. The DGKα-N-terminal region interacts with DGKα-C1 domains. (A) EGFP-DGKα-C-terminal region (*CTR*), -C1 domains (*C1Ds*) and -catalytic region (*CR*) were co-precipitated with GST alone or GST-DGKα-N-terminal region (*NTR*) in the absence of Ca^{2+} (with the addition of 5 mM EGTA). The precipitation of EGFP- and GST-tagged proteins was analyzed by Western blotting using anti-GST and anti-GFP antibodies. The data shown are representative of three independent experiments. *Input*: purified *GST alone* and *GST-DGKα-NTR* (upper panels) and COS-7 cell lysates expressing *EGFP-DGKα-CTR*, *-C1Ds* and *-CR* (lower panels). (B) The quantified relative intensities of the co-precipitated EGFP-DGKα-CTR, -C1Ds and -CR bands. The amount of EGFP-DGKα-CTR co-precipitated with GST-DGKα-NTR was set to 100%. The corresponding value of GST alone was subtracted. The error bars represent the standard deviation of three independent experiments. Statistical significance was determined using the student's *t*-test ($^{**}P < 0.01$).

2.2. The EF-hand motifs of DGKα interact with the C-terminal region of the enzyme

We next attempted to narrow down the interaction region at the N-terminal region of DGKα, which contains the RVH domain and EF-hand motifs. To this end, in addition to the GST-DGKα-N-terminal region, the RVH domain and the EF-hand motifs of DGKα (Fig. 1) were separately fused with GST, expressed in *Escherichia coli* cells and purified (Fig. S2). These proteins showed the expected molecular weights, 38 K and 36 K, respectively (Fig. S2). We confirmed that the GST-DGKα-N-terminal region was co-precipitated with EGFP-DGKα-C-terminal region (Fig. 3). The bands of the RVH domain and the EF-hand motifs that were pulled down were weaker than that of the N-terminal region. However, compared with the RVH domain, the EF-hand motifs exhibited markedly stronger interactions with the C-terminal region, and the observed band was an approximately three times more intense (Fig. 3). This result indicates that the EF-hand motifs of DGKα mainly contribute to the interaction with the C-terminal region of the enzyme.

2.3. EF-hand motifs of DGKα interact with the C1 domains of the enzyme

Because the N-terminal and C-terminal regions were used as partners of the C1 domains and EF-hand motifs in Figs. 2 and 3, respectively, we next confirmed that the EF-hand motifs of DGKα indeed associated with the C1 domains of the enzyme. We performed a co-precipitation analysis using the purified GST-fused DGKα-EF hand motifs and lysates of COS-7 cells expressing the EGFP-DGKα-C1 domains. As shown in Fig. 4, the DGKα-EF hand motifs co-precipitated with the EGFP-DGKα-C1 domains in the absence of Ca^{2+}.

Because the EF-hand motifs of DGKα are known to bind to Ca^{2+} ($K_d \approx 300$ nM) [26,27], we next attempted to determine whether the physical interaction between the EF-hand motifs and the C1 domains of DGKα was regulated by Ca^{2+}. Adding 1 μM Ca^{2+} to the co-precipitation mixture markedly attenuated the co-precipitation of the EGFP-DGKα-C1 domain with the GST-DGKα-EF-hand motifs, with an approximate 100% decrease (Fig. 4). The result demonstrated that Ca^{2+} induced the dissociation of the physical interaction between the DGKα-EF hand motifs and the DGKα-C1 domains, and supports the previously suggested model that Ca^{2+}-induced conformational changes of the EF-hand-containing

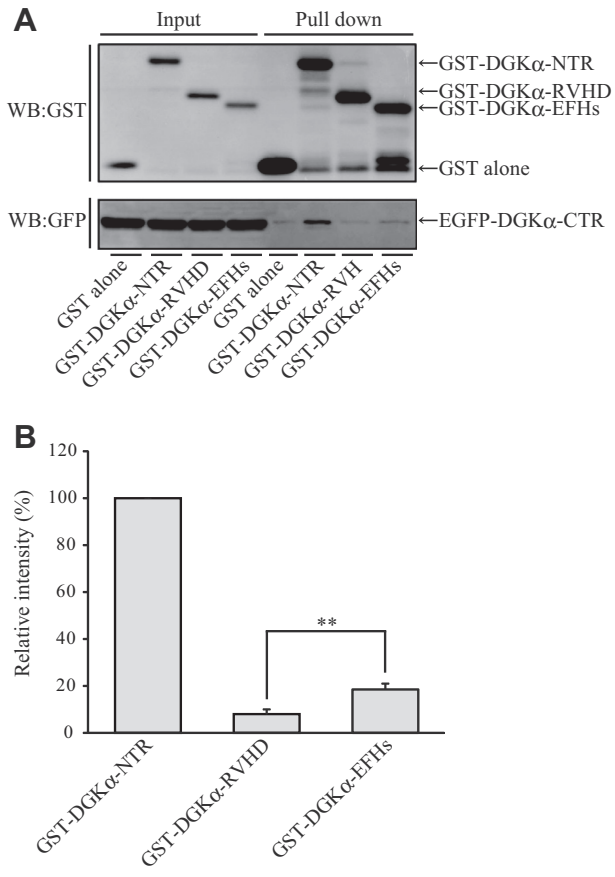

Fig. 3. The DGKα-C-terminal region interacts with DGKα-EF-hand motifs. (A) EGFP-DGKα-C-terminal region (*CTR*) was co-precipitated with GST alone, GST-DGKα-N-terminal region (*NTR*), GST-DGKα-RVH domain (*RVHD*) or GST-DGKα-EF hand motifs (*EFHs*) in the absence of Ca^{2+} (with the addition of 5 mM EGTA). The precipitation of EGFP- and GST-tagged proteins was analyzed by Western blotting using anti-GFP and anti-GST antibodies. The data shown are representative of three independent experiments. *Input*: purified *GST alone*, *GST-DGKα-NTR*, *-RVHD* and *-EFHs* (upper panel) and COS-7 cell lysates expressing *EGFP-DGKα-CT* (lower panel). (B) The quantified relative intensities of the co-precipitated GST-DGKα-NTR, GST-DGKα-RVHD and GST-DGKα-EFHs bands. The amount of GST-DGKα-NTR co-precipitated with EGFP-DGKα-CTR was set to 100%. The value of GST alone was subtracted. The error bars represent the standard deviation of three independent experiments. Statistical significance was determined using the student's *t*-test (**$P < 0.01$).

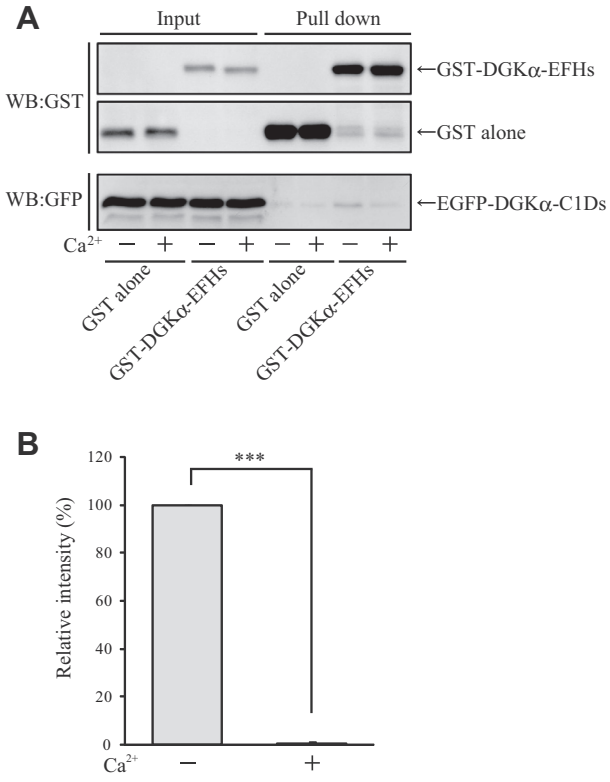

Fig. 4. DGKα-EF-hand motifs interact with DGKα-C1 domains. (A) GST alone or GST fused with DGKα-EF hand motifs (*EFHs*) was co-precipitated with EGFP-DGKα-C1 domains (*C1Ds*) in the absence (with the addition of 5 mM EGTA) or presence of Ca^{2+} (1 μM free-Ca^{2+}, adjusted by adding EGTA/Ca^{2+} solution as calculated using Calcon software). The precipitation of EGFP- and GST-tagged proteins was analyzed by Western blotting using anti-GFP and anti-GST antibodies. The data shown are representative of three independent experiments. *Input*: purified *GST alone* and *-EFHs* (upper panels) and COS-7 cell lysates expressing *EGFP-DGKα-C1Ds* (lower panel). (B) The quantified relative intensities of the EGFP-DGKα-C1Ds co-precipitated with GST-DGKα-EFHs in the absence or the presence of Ca^{2+}. The amount of EGFP-DGKα-C1Ds in the absence of Ca^{2+} was set to 100%. The corresponding value of GST alone was subtracted. The error bars represent the standard deviation of three independent experiments. Statistical significance was determined using the student's *t*-test (***$P < 0.005$).

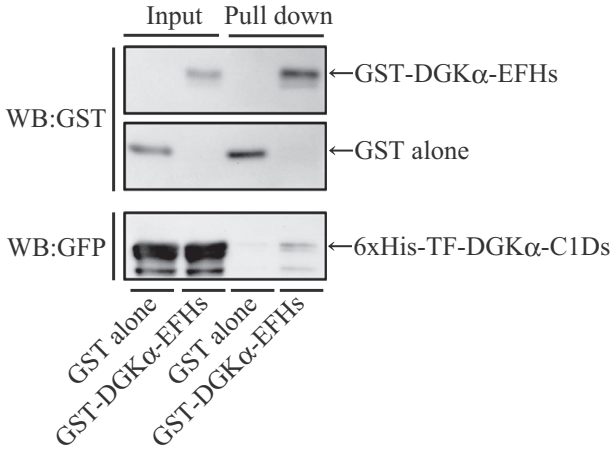

Fig. 5. Purified DGKα-C1 domains interact with purified DGKα-EF-hand motifs. (A) 6xHis-TF-fused DGKα-C1 domains (*C1Ds*) were expressed in bacteria and purified. GST alone or GST fused with DGKα-EF hand motifs (*EFHs*) was co-precipitated with 6xHis-TF-DGKα-C1Ds in the absence of Ca^{2+} (5 mM EGTA). The precipitation of 6xHis-TF- and GST-tagged proteins was analyzed by Western blotting using anti-6xHis and anti-GST antibodies. A representative result of three independent experiments is shown. *Input*: purified *GST alone* and *-EFHs* (upper panels) and purified *6xHis-TF-DGKα-C1Ds* (lower panel).

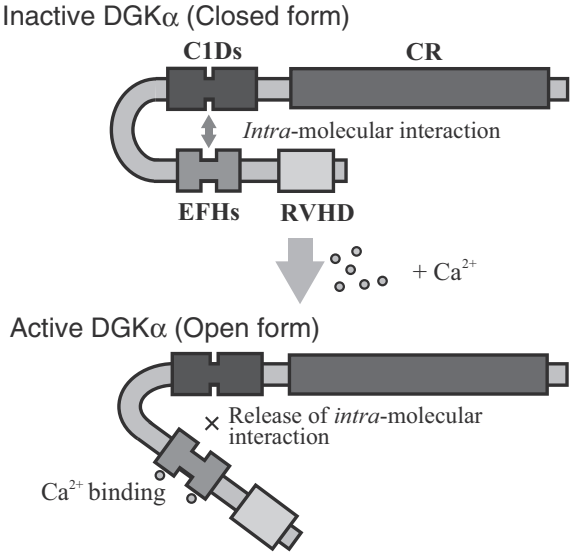

Fig. 6. A schematic representation of the proposed intra-molecular interaction and activation mechanisms of DGKα. The EF-hand motifs interact with the C1 domains. The N-terminal region, consisting of the RVH domain and the EF-hand motifs, sterically masks the C-terminal region of the enzyme. Elevated levels of Ca^{2+} trigger a conformational change that uncovers the C1 and catalytic domains. RVHD, recoverin homology domain; EFHs, EF-hand motifs; C1Ds, C1 domains; CR, catalytic region.

N-terminal region of DGKα unmask the DGKα-C-terminal region containing the C1 domains and the catalytic region [28–30,32].

Because the C1 domains of DGKα were expressed in COS-7 cells and were not purified, we cannot exclude the possibility that contaminating factors may have affected the interaction between the C1 domains and the EF-hand motifs of the enzyme. Therefore, we next attempted to confirm that highly purified DGKα C1 domains were able to bind to the DGKα EF-hand motifs that we had previously purified (Fig. S2). The bacterially expressed, cysteine-rich C1 domain has not been successfully purified so far because this domain is insoluble and is recovered in inclusion bodies. To circumvent the problems relating to insolubility, we employed a cold shock-trigger factor (TF) expression system [33], and 6xHis-TF-fused C1 domains of DGKα were successfully expressed and purified (Fig. S3). It was confirmed that the purified 6xHis-TF-C1 domains had the expected molecular weight, 70 K (Fig. S3). As shown in Fig. 5, purified GST-DGKα-EF hand motifs clearly co-precipitated with purified 6xHis-TF-DGKα-C1 domains, indicating that these two domains directly bind to each other.

3. Discussion

In this study, we demonstrated for the first time that the EF-hand motifs of DGKα interacted intra-molecularly with its C1 domains (Figs. 2–5). Moreover, the highly purified C1 domains were pulled down with the highly purified EF-hand motifs (Fig. 5), indicating that these domains directly interact with each other. We previously proposed the mechanisms by which DGKα is activated [32]. In that model, the N-terminal region of DGKα, consisting of the RVH domain and the EF-hand motifs, sterically masks the C-terminal region of the enzyme, consisting of the C1 and catalytic domains. In the presence of elevated levels of Ca^{2+}, a conformational change that uncovers the C1 and catalytic domains is trigger. In the new model, it is clear that the EF-hand motifs interact with the C1 domains (Fig. 6) and that the interaction between them is Ca^{2+}-sensitive (Fig. 5). Therefore, we have further refined the knowledge regarding the mechanism by which DGKα is activation.

In this study, we identified the EF-hand motifs of DGKα as a new target of the C1 domain. The C1 domain is included in a wide variety of important proteins, such as conventional and novel protein kinase Cs, Ras guanyl nucleotide-releasing protein, Unc-13 and chimaerin, and is known well to bind to DG and phorbol ester [6,7]. However, the protein target of C1 domain has not been clearly elucidated. With regard to its protein target, we previously reported that the N-terminal region of β2-chimaerin containing Src homology 2 and C1 domains, interacted with DGKγ [34]. However, the C1 domain alone did not bind to the catalytic region of DGKγ. The C1 domain in β2-chimaerin is located at the core of the structure, rather than being exposed, with the putative membrane-binding hydrophobic residues being occluded by intra-molecular contacts with other regions of the protein [35]. The present study showed that the C1 domain of DGKα bound intra-molecularly to the EF-hand motifs of the enzyme and that a Ca^{2+}-sensitive interaction should regulate the activity of the enzyme (Figs. 2–5). Our results expand the knowledge regarding the target and function of C1 domain.

This study provides several new insights into the functions and regulatory mechanisms of EF-hand-containing proteins. First, although EF-hand-containing proteins, such as calmodulin [36], calpain [23,24], calcineurin regulation subunit B [37] and ALG-2 [38] bind the cofactors, Ca^{2+}/calmodulin dependent protein kinase II, calpain small subunit 1, calcineurin and annexin XI, respectively, in an inter-molecular fashion, this study is, to our knowledge, the first demonstration that the EF-hand motifs, instead of the N-terminal region of DGKα containing the RVH domain and EF-hand motifs [32], can participate in an intra-molecular association. Second, we identified the C1 domains of DGKα as a new target of the EF-hand motif. Therefore, we expanded the binding protein list of the EF-hand motif.

Compared with the N-terminal region of DGKα, the EF-hand motifs alone and the RVH domain alone exhibited weaker interaction activities (Fig. 3). The result suggests that both the EF-hand motifs and the RVH domain are necessary to achieve the maximum binding activity with the C-terminal region. However, the EF-hand motifs exhibited markedly stronger interactions with the C-terminal region, corresponding to an approximately thrice

strong intensity, than the RVH domain. This indicates that EF-hand motifs are mainly responsible for the binding to the C1 domains. Because Jiang et al. [30] previously reported that the EF-hand motifs and RVH domain act as a functional unit during the Ca^{2+}-induced activation of DGKα, the RVH domain may sterically mask the catalytic site of the catalytic region.

In this study, we have shown that DGKα is quite unique among the EF-hand-containing proteins. Furthermore, our identification of a direct, intra-molecular, interaction between the EF-hand motifs and C1 domains of DGKα helps elucidate the activation mechanism of this pathophysiologically important enzyme [15]. However, further studies, including the determination of the tertiary structure of DGKα, are needed to explore the regulation of the activity of the enzyme in greater detail.

4. Experimental procedures

4.1. Plasmid constructs

The pGEX-6P-1-DGKα-N-terminal region (amino acids (aa) 1–200) construct (Fig. 1) was generated as previously described [32]. The pEGFP-DGKα-C-terminal region (aa 197–734) construct was prepared as previously described [39]. The cDNAs encoding the DGKα-RVH domain (aa 1–110) and DGKα-EF-hand motifs (aa 103–200) were generated from porcine DGKα-cDNA [11] and subcloned into pGEX-6P-1 (GE Healthcare Bio-Sciences, Tokyo, Japan) at the *Eco*RI/*Xho*I site. The cDNAs encoding the DGKα-C1 domains (aa 196–362) and DGKα-catalytic region (aa 363–734) were generated from porcine DGKα-cDNA and subcloned into pEGFP-C3 at the *Hind*III/*Pst*I site. The cDNA encoding the DGKα-C1 domains (aa 196–362) was also subcloned into pCold TF DNA (Takara Bio, Otsu, Japan) at the *Hind*III/*Pst*I site.

4.2. Expression and purification of GST fusion proteins

BL21 cells were transformed with the pGEX-6P-1 constructs. GST alone and GST-fusion proteins were expressed and purified according to the procedure recommended by the manufacturer (GE Healthcare Bio-Sciences). Specifically, the expression of fusion proteins was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Wako Pure Chemical Industries) at 37 °C for 3 h. The cells were then lysed by sonication in 50 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 1% (V/V) Triton X-100 (Nacalai Tesque, Kyoto, Japan), 1 mM EDTA (Dojindo, Kumamoto, Japan), 1 mM dithiothreitol, 20 μg/ml aprotinin (Wako Pure Chemical Industries), 20 μg/ml leupeptin (Nacalai Tesque), 20 μg/ml pepstatin (Nacalai Tesque), 20 μg/ml soybean trypsin inhibitor (Wako Pure Chemical Industries) and 1 mM phenylmethylsulfonyl fluoride (Wako Pure Chemical Industries). The insoluble material was removed by centrifugation. The supernatants were purified by affinity chromatography on a glutathione–Sepharose 4B column (GE Healthcare Bio-Sciences) at 4 °C. The purified proteins were dialyzed in phosphate-buffered saline containing 5 mM ethylene glycol tetraacetic acid (EGTA) (Dojindo).

4.3. Expression and purification of 6xHis-TF fusion protein

BL21 cells were transformed with the pCold-TF-DNA constructs. The TF-fusion proteins were expressed and purified according to the procedure recommended by the manufacturer (Takara Bio). Specifically, the expression of fusion proteins was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 15 °C for 24 h. The cells were then lysed by sonication in 50 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 1% (V/V) Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin, 20 μg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride. The insoluble material was removed by centrifugation. The supernatants were purified by affinity chromatography on a Ni Sepharose 6 fast flow column (GE Healthcare) at 4 °C. The purified proteins were dialyzed in Tris–HCl buffer (pH 7.4) containing 5 mM EGTA.

4.4. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. The cells were transfected with the cDNAs by electroporation (1×10^6 cells/2 mm gap cuvette, 110 V, 20.0 ms pulse length, one pulse) with the Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer's instructions.

4.5. In vitro binding assay

COS-7 cells ($\sim 1 \times 10^7$ cells/100-mm dish) expressing either enhanced green fluorescent protein (EGFP) alone or EGFP-tagged DGKα-CTR were lysed in 1 ml of 50 mM HEPES, pH 7.2, 1% (V/V) Nonidet P-40 (MP Biomedicals, Tokyo, Japan), 5 mM EGTA, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor mixture (1 tablet/50 ml, Roche Molecular Biochemicals, Tokyo, Japan). The mixture was centrifuged at 12,000g for 10 min at 4 °C. The resulting cell lysates (500 μl each) were incubated with 10 μg of GST and GST fusion proteins for 1 h at 4 °C. Then, 10 μl of glutathione–Sepharose beads were added to the lysates, and the mixture was incubated for 30 min at 4 °C with constant rocking. The beads were washed four times with 50 mM HEPES, pH 7.2, 0.1% (V/V) Triton X-100, 0.5 mM EGTA, 100 mM NaCl, 5 mM $MgCl_2$ and 10% glycerol. The washed beads were boiled in 50 μl SDS sample buffer. The total lysates and precipitates were analyzed by Western blotting using anti-GST and anti-GFP monoclonal antibodies as described below.

One hundred fifty picomoles TF-fused DGKα-C1 domains was incubated with 150 pmol of GST and GST fusion proteins in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 5 mM EGTA for 1 h at 4 °C. Then, 10 μl of glutathione–Sepharose beads were added to the mixture, and the samples were incubated for 30 min at 4 °C with constant rocking. The beads were washed three times with 50 mM HEPES, pH 7.2, 0.1% (V/V) Triton X-100, 0.5 mM EGTA, 150 mM NaCl, 5 mM $MgCl_2$ and 10% glycerol. The washed beads were boiled in 50 μl SDS sample buffer. The total lysates and precipitates were analyzed by Western blotting using anti-GST and anti-His tag monoclonal antibodies as described below.

4.6. Western blot analysis

The cell lysates and immunoprecipitates were separated using SDS–PAGE. The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Tokyo, Japan) and blocked with 5% (w/w) skim milk. The membrane was incubated with anti-GST monoclonal antibody (B-14, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFP monoclonal antibody (B-2, Santa Cruz Biotechnology) or anti-6xHis monoclonal antibody (9C11, Wako Pure Chemical Industries) in 5% skim milk for 1 h. The immunoreactive bands were visualized using peroxidase-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and the ECL Western blotting detection system (GE Healthcare Bio-Sciences).

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Japan Science and Technology Agency; the Naito Foundation; the Hamaguchi Foundation for the Advancement of Biochemistry; the Daiichi-Sankyo Foundation of Life Science; the Terumo Life Science Foundation; the Futaba Electronic Memorial Foundation; the Daiwa Securities Health Foundation; the Ono Medical Research Foundation; the Japan Foundation for Applied Enzymology; and the Food Science Institute Foundation.

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.fob.2014.04.003.

References

[1] Goto, K., Hozumi, Y. and Kondo, H. (2006) Diacylglycerol, phosphatidic acid, and the converting enzyme, diacylglycerol kinase, in the nucleus. Biochim. Biophys. Acta 1761, 535–541.
[2] Merida, I., Avila-Flores, A. and Merino, E. (2008) Diacylglycerol kinases: at the hub of cell signalling. Biochem. J. 409, 1–18.
[3] Sakane, F., Imai, S., Kai, M., Yasuda, S. and Kanoh, H. (2007) Diacylglycerol kinases: why so many of them? Biochim. Biophys. Acta 1771, 793–806.
[4] Shulga, Y.V., Topham, M.K. and Epand, R.M. (2011) Regulation and functions of diacylglycerol kinases. Chem. Rev. 111, 6186–6208.
[5] van Blitterswijk, W.J. and Houssa, B. (2000) Properties and functions of diacylglycerol kinases. Cell Signal. 12, 595–605.
[6] Brose, N. and Rosenmund, C. (2002) Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. J. Cell Sci. 115, 4399–4411.
[7] Colon-Gonzalez, F. and Kazanietz, M.G. (2006) C1 domains exposed: from diacylglycerol binding to protein–protein interactions. Biochim. Biophys. Acta 1761, 827–837.
[8] English, D. (1996) Phosphatidic acid: a lipid messenger involved in intracellular and extracellular signalling. Cell Signal. 8, 341–347.
[9] Exton, J.H. (1994) Phosphatidylcholine breakdown and signal transduction. Biochim. Biophys. Acta 1212, 26–42.
[10] Hodgkin, M.N., Pettitt, T.R., Martin, A., Michell, R.H., Pemberton, A.J. and Wakelam, M.J. (1998) Diacylglycerols and phosphatidates: which molecular species are intracellular messengers? Trends Biochem. Sci. 23, 200–204.
[11] Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C. and Tanabe, T. (1990) Porcine diacylglycerol kinase sequence has zinc finger and E-F hand motifs. Nature 344, 345–348.
[12] Yamada, K., Sakane, F. and Kanoh, H. (1989) Immunoquantitation of 80 kDa diacylglycerol kinase in pig and human lymphocytes and several other cells. FEBS Lett. 244, 402–406.
[13] Goto, K., Watanabe, M., Kondo, H., Yuasa, H., Sakane, F. and Kanoh, H. (1992) Gene cloning, sequence, expression and in situ localization of 80 kDa diacylglycerol kinase specific to oligodendrocyte of rat brain. Mol. Brain Res. 16, 75–87.
[14] Yanagisawa, K., Yasuda, S., Kai, M., Imai, S., Yamada, K., Yamashita, T., Jimbow, K., Kanoh, H. and Sakane, F. (2007) Diacylglycerol kinase α suppresses tumor necrosis factor-α-induced apoptosis of human melanoma cells through NF-κB activation. Biochim. Biophys. Acta. 1771, 462–474.
[15] Sakane, F., Imai, S., Kai, M., Yasuda, S. and Kanoh, H. (2008) Diacylglycerol kinases as emerging potential drug targets for a variety of diseases. Curr. Drug Targets 9, 626–640.
[16] Flores, I., Jones, D.R., Cipres, A., Diaz-Flores, E., Sanjuan, M.A. and Merida, I. (1999) Diacylglycerol kinase inhibition prevents IL-2-induced G1 to S transition through a phosphatidylinositol-3 kinase-independent mechanism. J. Immunol. 163, 708–714.
[17] Olenchock, B.A., Guo, R., Carpenter, J.H., Jordan, M., Topham, M.K., Koretzky, G.A. and Zhong, X.P. (2006) Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. Nat. Immunol. 7, 1174–1181.
[18] Zha, Y., Marks, R., Ho, A.W., Peterson, A.C., Janardhan, S., Brown, I., Praveen, K., Stang, S., Stone, J.C. and Gajewski, T.F. (2006) T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-α. Nat. Immunol. 7, 1166–1173.
[19] Cutrupi, S., Baldanzi, G., Gramaglia, D., Maffe, A., Schaap, D., Giraudo, E., van Blitterswijk, W., Bussolino, F., Comoglio, P.M. and Graziani, A. (2000) Src-mediated activation of alpha-diacylglycerol kinase is required for hepatocyte growth factor-induced cell motility. EMBO J. 19, 4614–4622.
[20] Takeishi, K., Taketomi, A., Shirabe, K., Toshima, T., Motomura, T., Ikegami, T., Yoshizumi, T., Sakane, F. and Maehara, Y. (2012) Diacylglycerol kinase alpha enhances hepatocellular carcinoma progression by activation of Ras-Raf-MEK-ERK pathway. J. Hepatol. 57, 77–83.
[21] Kretsinger, R.H. (1979) The informational role of calcium in the cytosol. Adv. Cyclic Nucleotide Res. 11, 1–26.
[22] Moncrief, N.D., Kretsinger, R.H. and Goodman, M. (1990) Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. J. Mol. Evol. 30, 522–562.
[23] Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. and Suzuki, K. (1984) Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? Nature 312, 566–570.
[24] Murachi, T. (1984) Calcium-dependent proteinases and specific inhibitors: calpain and calpastatin. Biochem. Soc. Symp. 49, 149–167.
[25] Bairoch, A. and Cox, J.A. (1990) EF-hand motifs in inositol phospholipid-specific phospholipase C. FEBS Lett. 269, 454–456.
[26] Sakane, F., Yamada, K., Imai, S. and Kanoh, H. (1991) Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium-dependent translocation. J. Biol. Chem. 266, 7096–7100.
[27] Yamada, K., Sakane, F., Matsushima, N. and Kanoh, H. (1997) EF-hand motifs of α, β and γ isoforms of diacylglycerol kinase bind calcium with different affinities and conformational changes. Biochem. J. 321, 59–64.
[28] Merino, E., Sanjuan, M.A., Moraga, I., Cipres, A. and Merida, I. (2007) Role of the diacylglycerol kinase alpha-conserved domains in membrane targeting in intact T cells. J. Biol. Chem. 282, 35396–35404.
[29] Sakane, F., Imai, S., Yamada, K. and Kanoh, H. (1991) The regulatory role of EF-hand motifs of pig 80 K diacylglycerol kinase as assessed using truncation and deletion mutants. Biochem. Biophys. Res. Commun. 181, 1015–1021.
[30] Jiang, Y., Qian, W., Hawes, J.W. and Walsh, J.P. (2000) A domain with homology to neuronal calcium sensors is required for calcium-dependent activation of diacylglycerol kinase alpha. J. Biol. Chem. 275, 34092–34099.
[31] Yamada, K. and Kanoh, H. (1988) Occurrence of immunoreactive 80 kDa and non-immunoreactive diacylglycerol kinases in different pig tissues. Biochem. J. 255, 601–608.
[32] Takahashi, M., Yamamoto, T., Sakai, H. and Sakane, F. (2012) Calcium negatively regulates an intramolecular interaction between the N-terminal recoverin homology and EF-hand motif domains and the C-terminal C1 and catalytic domains of diacylglycerol kinase α. Biochem. Biophys. Res. Commun. 423, 571–576.
[33] Qing, G., Ma, L.C., Khorchid, A., Swapna, G.V., Mal, T.K., Takayama, M.M., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, G.T., Ikura, M. and Inouye, M. (2004) Cold-shock induced high-yield protein production in *Escherichia coli*. Nat. Biotechnol. 22, 877–882.
[34] Yasuda, S., Kai, M., Imai, S.I., Kanoh, H. and Sakane, F. (2008) Phorbol ester and hydrogen peroxide synergistically induce the interaction of diacylglycerol kinase γ with the Src homology 2 and C1 domains of β2-chimaerin. Biochem. J. 409, 95–106.
[35] Canagarajah, B., Leskow, F.C., Ho, J.Y., Mischak, H., Saidi, L.F., Kazanietz, M.G. and Hurley, J.H. (2004) Structural mechanism for lipid activation of the Rac-specific GAP, beta2-chimaerin. Cell 119, 407–418.
[36] Stratton, M.M., Chao, L.H., Schulman, H. and Kuriyan, J. (2013) Structural studies on the regulation of Ca^{2+}/calmodulin dependent protein kinase II. Curr. Opin. Struct. Biol. 23, 292–301.
[37] Li, J., Jia, Z., Zhou, W. and Wei, Q. (2009) Calcineurin regulatory subunit B is a unique calcium sensor that regulates calcineurin in both calcium-dependent and calcium-independent manner. Proteins 77, 612–623.
[38] Satoh, H., Shibata, H., Nakano, Y., Kitaura, Y. and Maki, M. (2002) ALG-2 interacts with the amino-terminal domain of annexin XI in a Ca^{2+}-dependent manner. Biochem. Biophys. Res. Commun. 291, 1166–1172.
[39] Tsushima, S., Kai, M., Yamada, K., Imai, S., Houkin, K., Kanoh, H. and Sakane, F. (2004) Diacylglycerol kinase γ serves as an upstream suppressor of Rac1 and lamellipodium formation. J. Biol. Chem. 279, 28603–28613.