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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

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ABSTRACT

The type I diacylglycerol kinase (DGK) isozymes (α, β and γ) contain a shared recoverin homology (RVH) domain, a tandem repeat of Ca^{2+}-binding EF-hand motifs, two cysteine-rich C1 domains, and the catalytic domain. We previously reported that a DGKα mutant lacking the RVH domain and EF-hands was constitutively active, implying that the N-terminal region (NTR) of DGKα, consisting of the RVH domain and EF-hand motifs, intramolecularly interacts with and masks the activity of the C-terminal region (CTR), containing the C1 and catalytic domains. In this study, we demonstrate that a glutathione S-transferase (GST)-fused DGKα-NTR construct physically binds to a green fluorescent protein (GFP)-fused DGKα-CTR construct. Moreover, co-precipitation of GFP-DGKα-CTR with GST-DGKα-NTR was clearly attenuated by the addition of 1 μM Ca^{2+}. This result indicates that Ca^{2+} induces dissociation of the physical interaction between DGKα-NTR and DGKα-CTR. In addition to previously reported calcium-dependent changes in the hydrophobicity and net surface charge, Ca^{2+} also appeared to induce a decrease in the α-helical content of DGKα-NTR. These results suggest that Ca^{2+}-induced conformational changes in the NTR release the intramolecular association between the NTR and the CTR of DGKα.

1. Introduction

Diacylglycerol kinase (DGK) converts diacylglycerol to phosphatidic acid [1–5]. Diacylglycerol activates protein kinase C, chimaerins, Unc-13 and Ras guanyl nucleotide-releasing protein. Phosphatidic acid regulates a number of signaling proteins, such as phosphatidylinositol-4-phosphate 5-kinase, Ras GTPase-activating protein, Raf-1 kinase, and atypical PKC.

Ten mammalian DGK isozymes (α, β, γ, δ, ε, ζ, η, θ, ι and κ) have been identified. All of the isozymes share two or three characteristic cysteine-rich C1 domains and the catalytic region, and they have been subdivided into five groups according to their additional structural features [1–5]. Type I DGK isozymes (DGKs α, β and γ) 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 hands, 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 [1–5]. Interestingly, these isozymes exhibit different tissue- and cell-specific modes of expression despite the similarity of their structures. Specifically, DGKα is most abundant in T-lymphocytes and the thymus [6,7], oligodendrocytes of brain [8] and melanoma cells [9]. DGKα participates in interleukin-2-dependent T-cell proliferation [10], T-cell anergy [11,12], hepatocyte growth factor-induced cell motility [13], melanoma apoptosis [9] and the progression of human hepatocellular carcinoma [14].

Calcium-mediated cellular signal transduction mediated plays a pivotal role in the regulation of the physiological functions of various types of cells [15,16]. Calmodulin (CaM) is characteristic of many Ca^{2+}-binding proteins that have EF-hand structures. Most of the EF-hand proteins, such as CaM, troponin C and calcineurin regulation subunit B, are relatively small molecules (10–20 kDa) [16]. Such proteins play a specialized role as Ca^{2+}-sensitive regulators of many target enzymes, and their amino acid sequences consist primarily of EF-hand motifs. In contrast, type I DGKs are relatively large for EF-hand proteins (80–90 kDa) and represent a fusion protein composed of EF-hands combined with other functional domains [1–5]. Calcium-activated neutral protease, calpain [17,18] and inositol phospholipid-specific phospholipase C [19] are the only known examples of this type of fusion protein with a proven capacity for Ca^{2+}.

Abbreviations: CaM, calmodulin; CD, circular dichroism; CTR, C-terminal region of DGKα, consisting of the C1 and catalytic domains; DGK, diacylglycerol kinase; EDTA, ethylene diamine tetraacetic acid; EGFP, enhanced green fluorescent protein; EGTA, ethylene glycol tetraacetic acid; GST, glutathione S-transferase; NTR, N-terminal region of DGKα, consisting of the RVH domain and the EF-hand motifs; RVH, recoverin homology.

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Previously, we demonstrated that DGKα purified from pig thymus cytosol binds Ca^{2+} in 2:1 metal:enzyme stoichiometry using both EF-hand motifs with an apparent dissociation constant, K_d, of 300 nM [20,21]. Adding Ca^{2+} in the presence of phosphatidylserine significantly activates both the enzyme purified and the in situ enzyme expressed in COS-7 cells [20]. Interestingly, a DGKα mutant lacking the RVH and the EF-hand motif domains translocated from the cytosol to membranes [22,23] and became constitutively active [22–24]. Jiang et al. [24] further showed that the EF-hand motifs and RVH domain act as a functional unit during Ca^{2+}-induced activation of DGKα. These results imply that the N-terminal region (NTR) of DGKα, consisting of the RVH domain and EF hands, interacts with the C-terminal region (CTR), consisting of the C1 and catalytic domains, intramolecularly and thereby masks its activity. Such an interaction, however, has not yet been observed. The activation mechanism of DGKα is therefore not yet fully understood, even though it is involved in a variety of significant pathophysiological events. In this study, by expressing the NTR and CTR of DGKα separately, we showed that the two domains physically interact and that this association is attenuated by Ca^{2+}. We also observed calcium-dependent changes in the α-helical content of the DGKα-NTR. These results suggest that Ca^{2+}-induced conformational changes in the DGKα-NTR disrupt the intramolecular association between the NTR and the CTR of the enzyme.

2. Materials and methods

2.1. Plasmid constructs

DGKα-NTR cDNA (amino acids (aa) 1–200) was generated from porcine DGKα-cDNA [6] and subcloned into pGEX-6P-1 (GE Healthcare) at the *Eco*RI/*Xho*I site. CaM cDNA was amplified from mouse brain cDNA and inserted into pGEX-6P-1 at the *Bam*HI/*Xho*I site. pEGFP-DGKα-CTR (aa 197–734) was prepared as described previously [25].

2.2. Expression and purification of glutathione S-transferase (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). Specifically, the expression of fusion proteins was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h. 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, and insoluble material was removed by centrifugation. The supernatants were purified by affinity chromatography on a glutathione-Sepharose 4B column (GE Healthcare) at 4 °C. The purified proteins were dialyzed in phosphate-buffered saline containing 5 mM EGTA (Dojindo).

2.3. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemicals) containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. Cells were transiently transfected with the cDNAs using Polyfect transfection reagent according to the manufacturer's instructions (Qiagen). Transfected cells were incubated for 24–48 h prior to further analysis.

2.4. In vitro binding assay

COS-7 cells (~1×10^7 cells/60 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), 5 mM ethylene glycol tetraacetic acid (EGTA), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor mixture (1 tablet/50 ml, Roche Molecular Biochemicals). The mixture was centrifuged at 12,000 x g 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. 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 4 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 blot using anti-FLAG and anti-GFP monoclonal antibodies as described below.

2.5. Western-Blot analysis

Cell lysates and immunoprecipitates were separated using SDS–PAGE. The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories) and blocked with Block Ace (Dainippon Pharmaceutical). The membrane was incubated with anti-FLAG M2 monoclonal antibodies (Sigma–Aldrich) or anti-GFP monoclonal antibodies (B-2, Santa Cruz Biotechnology) in 10% Block Ace for 1 h. The immunoreactive bands were visualized using peroxidase-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories) and the ECL Western-Blotting detection system (GE Healthcare).

2.6. Ultraviolet circular dichroism (CD) spectroscopy

The purified proteins (200 nM) were dissolved in a solution consisting of 10 mM Tris–HCl, pH 7.2 and 5 mM EGTA. The free Ca^{2+} concentrations in the mixtures were controlled by varying the EGTA (5 mM)/$CaCl_2$ ratio and calculated using Calcon software based on a report by Fabiato and Fabiato [26]. Far-ultraviolet CD measurements were performed using a JASCO J-805 spectrophotometer with a 1 cm quartz cuvette. The instrument was calibrated with ammonium camphor 10-sulfonate (concentration of 0.06% (w/v); at 290.5 nm, CD = 190.4 millidegrees). The mean residue ellipticity (θ) was measured, and the α-helical content was calculated from the value of θ according to Greenfield and Fasman [27]: fractional α-helicity = (θ_{208}–4000)/(33,000–4000).

3. Results

3.1. The N-terminal region of DGKα (DGKα-NTR), consisting of the RVH domain and the EF-hand motifs, physically interacts with the C-terminal region of the enzyme (DGKα-CTR), consisting of the C1 and catalytic domains

We produced GST, GST-fused with the DGKα-NTR (GST-DGKα-NTR) and GST-fused with CaM (GST–CaM) (Fig. 1A) by bacteria expression, followed by affinity purification using glutathione Sepharose beads. We obtained proteins with the expected molecular weights (GST: 26 K; GST-DGKα-NTR: 42 K; GST–CaM: 43 K) in high purity (Fig. 1B). The DGKα-CTR could not be expressed in *Escherichia coli* because the cysteine-rich C1 domain renders it insoluble. Instead, we expressed EGFP-tagged DGKα-CTR in mammalian COS-7 cells and obtained a protein with the expected molecular weight (85 K, Fig. 1C).

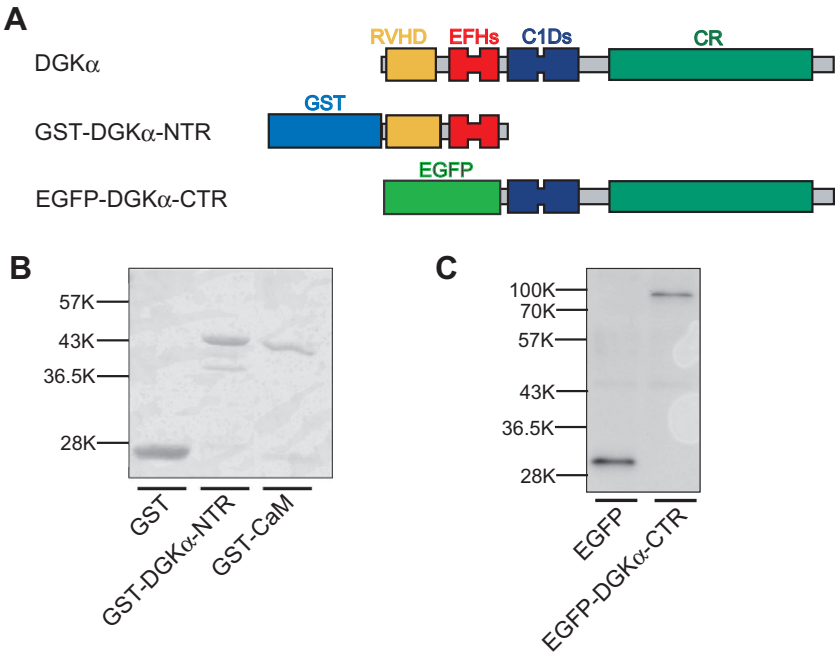

Fig. 1. Expression and purification of DGKα mutants used in this study. (A) Schematic representation of DGKα mutants used in this study. (B) GST-DGKα-NTR was purified by glutathione Sepharose column chromatography, separated by SDS–PAGE and stained with Coomassie Brilliant Blue. Purified GST alone and GST-CaM are also shown. (C) The expression of EGFP-DGKα-CTR and EGFP alone expressed in COS-7 cells was visualized by Western blotting using anti-GFP antibodies. RVHD, recoverin homology domain; EFHs, EF-hand motifs; C1Ds, C1 domains; CR, catalytic region.

We examined the physical interaction between the N-terminal region of DGKα (DGKα-NTR), consisting of the RVH domain and the EF hand motifs, and the C-terminal region of the enzyme (DGKα-CTR), consisting of the C1 and catalytic domains, by conducting a co-precipitation analysis using purified GST-fused DGKα-NTR and the lysates of COS-7 cells expressing EGFP-DGKα-CTR. When GST-DGKα-NTR was precipitated with glutathione beads in the absence of Ca^{2+}, EGFP-DGKα-CTR co-precipitated (Fig. 2A and B). EGFP alone did not co-precipitate with GST-DGKα-NTR (Fig. 2A), and GST alone did co-precipitate with EGFP-DGKα-CTR (Fig. 2B). When the same experiment was conducted with GST–CaM as a control, CaM failed to co-precipitate EGFP-DGKα-CTR (data not shown). These results indicate that the NTR of DGKα specifically interacts with the CTR of the enzyme. This result is consistent with the previously proposed hypothesis that the NTR of DGKα interacts with its CTR intramolecularly in the absence of Ca^{2+} [22–24].

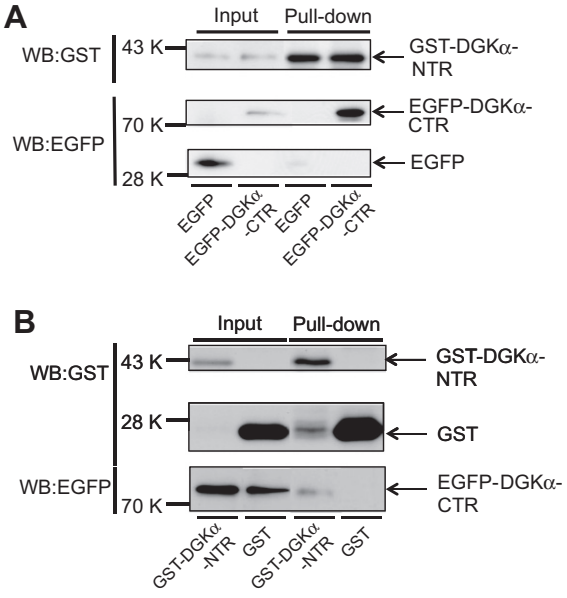

Fig. 2. DGKα-NTR interacts with DGKα-CTR. (A) EGFP and EGFP-DGKα-CTR co-precipitate with GST-DGKα-NTR in the absence of Ca^{2+} (5 mM EGTA). (B) EGFP-DGKα-CTR co-precipitates with both GST alone and GST-DGKα-NTR in the absence of Ca^{2+} (5 mM EGTA). Precipitation of EGFP- and GST-tagged proteins was analyzed by Western Blotting using anti-GST and anti-GFP antibodies. Data shown are representative of triplicate experiments.

3.2. Calcium negatively regulates the physical interaction between DGKα-NTR and DGKα-CTR

Because the EF-hand motifs of DGKα are known to bind to Ca^{2+} ($K_d \approx 300$ nM) [20,21], we next attempted to determine whether the physical interaction between DGKα-NTR and DGKα-CTR is regulated by Ca^{2+}. Adding of 1 μM Ca^{2+} to the co-precipitation mixture markedly attenuated the co-precipitation of EGFP-DGKα-CTR with GST-DGKα-NTR (an approximately 65% decrease, Fig. 3). The result demonstrates that Ca^{2+} induces dissociation of the physical interaction between DGKα-NTR and DGKα-CTR and supports the previously suggested model that Ca^{2+}-induced conformational changes of the EF-hand-containing NTR of DGKα unmask the catalytic region in the CTR of the enzyme [22–24].

3.3. Calcium-dependent changes in the α-helical content of DGKα-NTR

Previously, we reported that Ca^{2+} induced a decrease of hydrophobicity and an increase of positive net surface charge in the EF-hand motifs of DGKα [21]. We now examined whether the α-helical content of DGKα-NTR is also altered in the presence of Ca^{2+} using circular dichroism (CD) spectroscopy. The ultraviolet CD spectrum of an α-helix exhibits characteristic peaks at 208 and 222 nm (molar ellipticity (deg cm^2/dmol)). We confirmed that the ultraviolet CD spectrum of GST alone was not affected by Ca^{2+} (Fig. 4A). In contrast, the ellipticity of GST–CaM was notably

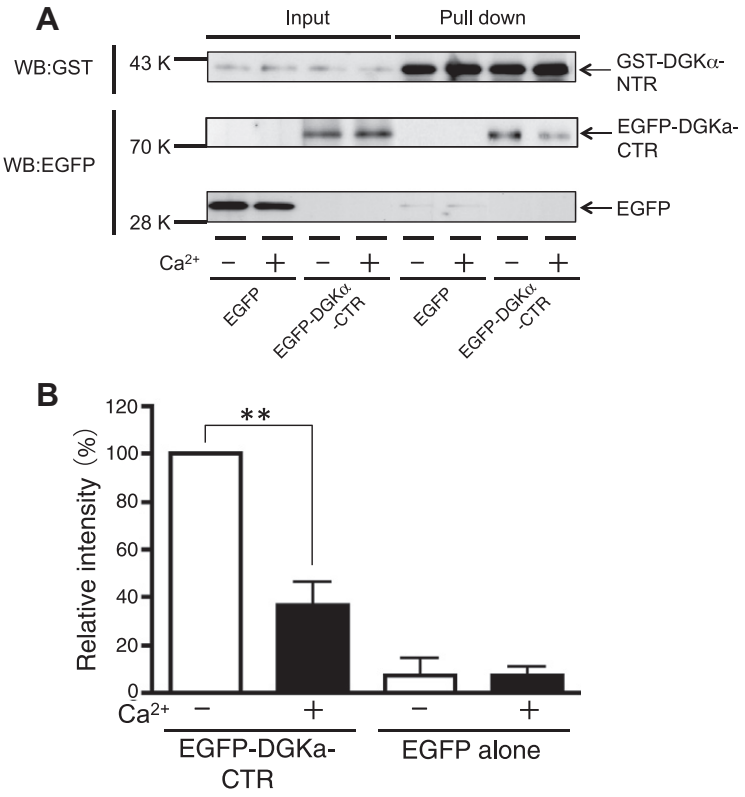

Fig. 3. Calcium negatively regulates the interaction between DGKα-NTR with DGKα-CTR. (A) EGFP and EGFP-DGKα-CTR co-precipitate with GST-DGKα-NTR in the absence (5 mM EGTA) or presence of Ca^{2+} (1 μM free-Ca^{2+}, adjusted by adding EGTA/Ca^{2+} solution as calculated using Calcon software). Precipitation of EGFP- and GST-tagged proteins was analyzed by Western Blotting using anti-GST and anti-GFP antibodies. Data shown are representative of triplicate experiments. (B) Quantified relative intensities of the co-precipitated EGFP-DGKα-CTR bands. Error bars represent standard deviation of three independent experiments. Statistical significance was determined using the student's t-test (**, $P < 0.01$).

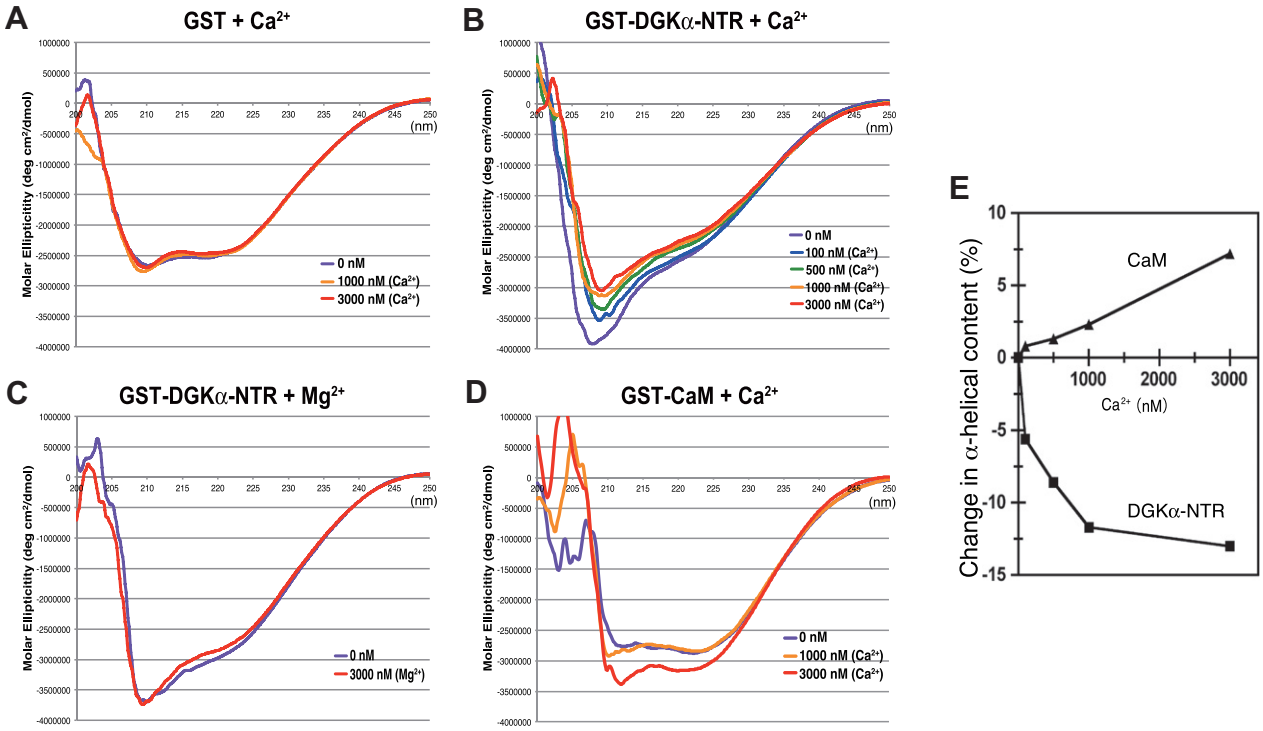

Fig. 4. Effect of calcium on the ultraviolet CD spectra of GST, GST-DGKα-NTR and GST-CaM. (A, B and D) CD spectra of 200 nM of GST (A), GST-DGKα-NTR (B) and GST-CaM (D) were measured in the presence of the following concentration of free-Ca^{2+}: 0, 100, 500, 1000 or 3000 nM. Only the spectra for 0, 1000 or 3000 nM of Ca^{2+} are exhibited in (A) and (D). The concentrations of free Ca^{2+} in each mixture were controlled by varying the ratio of EGTA (5 mM):$CaCl_2$. (C) CD spectra of 200 nM of GST-DGKα-NTR in the absence or presence of 3000 nM free-Mg^{2+}. The concentration of free Mg^{2+} was controlled by varying the ratio of EDTA (5 mM):$MgCl_2$. (E) Ca^{2+}-dependent changes in the α-helical content of DGKα-NTR and CaM are also shown. Data shown are representative of triplicate experiments.

increased in the presence of Ca^{2+} (Figs. 4D and E), indicating an increase of α-helical content, as previously described [28,29]. The intensity of the peaks at 208 and 222 nm in the CD spectrum of GST-DGKα-NTR, however, was clearly reduced by the addition of Ca^{2+} in a dose-dependent manner (Fig. 4B). This result indicates that the α-helical content of DGKα-NTR decreases in the presence of Ca^{2+}. The ED_{50} value for the Ca^{2+}-induced secondary structure change of DGKα was approximately 300 nM (Figs. 4B and E), which is comparable to the K_d value for Ca^{2+} binding to the enzyme (300 nM) and to the ED_{50} value for the Ca^{2+}-induced activation of the enzyme (340 nM) [20,21]. The addition of Mg^{2+} did not produce similar changes in the CD spectrum of the enzyme (Fig. 4C), strongly suggesting that the effect is Ca^{2+}-specific.

4. Discussion

Although it has been hypothesized that the NTR of DGKα, consisting of the RVH domain and the EF-hand motifs, masks the CTR of the enzyme, consisting of the C1 and catalytic domains, and that elevated levels of Ca^{2+} trigger a conformational change that uncovers the C1 and catalytic domains [22–24] (Suppl. Fig. 1), no such interaction has yet been reported. In this study, we have demonstrated, for the first time, that the NTR of DGKα does physically associate with the CTR (Fig. 2). Intriguingly, the association was attenuated by the addition of Ca^{2+} (Fig. 3). These results strongly support the hypothesis described above and provide useful information concerning the activation mechanism of DGKα. We also demonstrated that the NTR of DGKα underwent a Ca^{2+}-induced decrease in α-helical content (Fig. 4). This observation implies that, in addition to the previously described alterations in hydrophobicity [21] and net surface charge [21], a secondary structure change may play an important role in the activation mechanism of DGKα. The calcium-dependent disruption of the intramolecular association between the NTR and CTR of DGKα may be intimately involved in the regulation of its activation. We were unable to quantitatively determine the effect of DGKα-NTR on the activity of DGKα-CTR, however, because DGKα-NTR only partly (at most 10%) bound to DGKα-CTR (Fig. 2). Furthermore, any proximity effects or interaction resulting from the covalent bond that links the NTR and the CTR of in the intact enzyme were lost in this experiment because the NTR and CTR of DGKα were produced as separate polypeptides. The independently expressed NTR may affect the activity of the CTR to a significantly lesser extent than the same region in the intact enzyme.

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 calpain and inositol phospholipid-specific phospholipase C bind cofactors in an intermolecular fashion, this is, to our knowledge, the first demonstration that the EF-hand motifs can participate in an intra-molecular association. Second, the α-helical content of small EF-hand-containing proteins such as CaM (Fig. 4C and [28,29]), troponin C [28,30], calcineurin regulatory subunit B [31] and tescalcin [32] increased in the presence of calcium. Unexpectedly, this metal ion has an inverse effect on the α-helical content of the EF-hand-containing region of DGKα, the NTR. The secondary structure of calpain (an EF-hand-containing protease), another fusion protein composed of EF-hands and other functional domains, was not affected by Ca^{2+} [33]. Conformational changes of the EF-hands of inositol phospholipid-specific phospholipase C have not been reported. Third, CaM [34] and troponin C [35] undergo a conformational change that results in the exposure of hydrophobic amino acids upon binding Ca^{2+}. In contrast, the hydrophobic regions of the EF-hand motifs of DGKα were already exposed in the absence of Ca^{2+}, and the exposed hydrophobic area decreased in response to Ca^{2+}, as previously reported [21]. The Ca^{2+}-induced conformational changes of the EF-hand-containing DGKα-NTR are therefore quite unusual and novel.

In this study, we have shown that DGKα is unique among EF-hand-containing proteins. Furthermore, our identification of a physical, intramolecular, interaction between the NTR and CTR of DGKα helps elucidate the activation mechanism of this pathophysiologically important enzyme. Nevertheless, further studies, including determination of the tertiary structure of DGKα are needed to explore the regulation of the activity of the enzyme in greater detail.

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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.2012.06.006.

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