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Both the C1 domain and a basic amino acid cluster at the C-terminus are important for the neurite and branch induction ability of DGKβ

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ABSTRACT

We previously reported that diacylglycerol kinase β (DGKβ) induces neurites and branches, contributing to higher brain function including emotion and memories. However, the detailed molecular mechanism of DGKβ function remains unknown. Therefore, we constructed various mutants of DGKβ and compared their enzyme activity, intracellular localization, and ability to induce neurites and branching in SH-SY5Y cells.

Even when RVH-domain and EF-hand motif were deleted, the mutant showed similar plasma membrane localization and neurite induction compared to wild type (WT), although the kinase activity of the mutant was three times higher than that of WT. In contrast, further deletion of C1 domain reduced the activity to 50% and abolished plasma membrane localization and neurite induction ability. When 34 amino acids were deleted from C-terminus, the mutants completely lost enzyme activity, plasma membrane localization, and the ability to induce neurites. A kinase-negative mutant of DGKβ retained plasma membrane localization and induced significant neurites and branches; however, the rate of induction was weaker than that of WT. Furthermore, C1A and C1B mutants, which have a mutation in a cysteine residue in the C1A or C1B domain, and the RK/E mutant, which has substitutions of arginine and lysine to glutamic acid in a cluster of basic amino acids at the C-terminus, lost their plasma membrane localization and neurite induction ability. These results indicate that in addition to kinase activity, plasma membrane localization via the C1 domain and basic amino acids at the C-terminus were indispensable for neurite induction by DGKβ.

1. Introduction

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA). DG is an important lipid messenger that regulates several proteins, including protein kinase C (PKC), chimerins and Unc-13. PA is also an important signaling molecule that activates several enzymes, including the mammalian target of rapamycin (mTOR) and a typical PKC. Therefore, DGK is thought to be a key enzyme for numerous cellular responses [1–5]. Indeed, recent researches using DGK knockout (KO) mice clearly demonstrated importance of DGK in the immune system [6,7], its pathophysiological roles in brain and heart [7], and its role in insulin resistance in diabetes [8–10].

To date, ten subtypes of DGK have been cloned and categorized into five groups based on primary structural motifs. All DGKs, except DGKθ, have two cysteine-rich regions homologous to those of PKCs (C1A and C1B domains). These regions are located in regulatory domain at the N-terminal half, and a catalytic domain is present in the C-terminal half. DGKθ has three C1 domains and a separated catalytic domain. In addition, Type I DGKs (DGKα, β and γ) have an EF-hand and a recoverin homology (RVH) domain. Type II DGKs (DGKδ, η and κ) have a pleckstrin homology (PH) domain instead of EF-hand and RVH domains at the N-terminus. Type III DGK (DGKε) contains only the C1 domains. Type IV DGKs (DGKζ and ι) have a myristoylated alanine-rich C-kinase substrate-like region at the N-terminus and four ankyrin repeats at the C-terminus. Type V DGK (DGKθ) has a proline- and glycine-rich and PH domains.

DGKβ was cloned from a rat brain cDNA library in 1993 [11] and is predominantly localized in neurons, specifically in the cerebral

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cortex, hippocampus, and caudate–putamen [11–13]. The expression of DGKβ rapidly increases after 14 days of age [12], when synaptic maturation progresses. In addition, Caricasole et al. reported that one of the splice variants of human DGKβ, which lacks 35 amino acids at the C-terminus but has an additional 4-amino-acid extension (DGKβ SV3; GenBank accession number AX032745), is associated with a human DGKβ EST that is annotated as differentially expressed in patients with mood disorders [14]. These results suggest that DGKβ is important for neuronal functions and is related to neuronal diseases. Indeed, we produced DGKβ KO mice and showed that DGKβ regulates neurites, branches, and spines, and its loss causes impairment of emotion and long-term memory related to cognitive functions [15,16]. However, the precise molecular mechanisms of the DGKβ-induced morphological change remain unknown. Therefore, we constructed various mutants of DGKβ and compared their enzyme activity, intracellular localization, and ability to induce neurites and branches in SH-SY5Y cells.

2. Materials and methods

2.1. Materials

SY-SY5Y and COS-7 cells were purchased from RIKEN CELL BANK. The plasmids encoding DGKβ was previously described [16]. The anti-GFP antibody was made in house.

2.2. Methods

2.2.1. Cell culture

COS-7 cells were cultured in DMEM medium (Nacali tesque). SH-SY5Y cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) and 2 mM Glutamax. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2.2. Construction of plasmids encoding GFP-DGKβ and mutants

The constructs encoding rat DGKβ fused to GFP (DGKβFL) were previously described [16]. cDNA fragments encoding DGKβFL C-cut, IncABS, KD, CA, CA C-cut, ST/A and KR/E mutants were produced by PCR using DGKβFL as a template and primers listed in Supplemental table 1, and subcloned into pEGFP-C1 vector (Clontech). The fragment digested with BglII and BamHI from DGKβFL was subcloned into pEGFP-C3 vector for C-terminus.

To make KN and PPP/AAA mutants, the primers listed in Supplemental table 1 were phosphorylated by T4-kinase. Using the phosphorylated primers and GFP-DGKβFL (template), mutagenesis was performed as described by the manufacturer's protocol using a Excite PCR-based Site-directed Mutagenesis kit (STRATAGENE). For C1A and C1B mutants, mutagenesis was carried out using a Quick change II XL Site-directed Mutagenesis kit (STRATAGENE). The plasmid encoding the mutants was screened by restriction enzyme which was additionally employed. The sequence of all constructs was finally checked by sequencing.

2.2.3. Immunoblotting and kinase assay

Plasmids (32 μg) were electroporated into COS-7 cells using a Gene pulser (Bio-Rad, 975 μF, 220 mV). After being cultured, the harvested cells were resuspended in homogenizing buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 50 mM Tris–HCl, 200 μg/ml leupeptin, 1 mM phenylmethylsulfonyl-fluoride, 1% TritonX-100, pH 7.4) and sonicated (UD-210 TOMY; output 3, 15 s, 2 times).

For immunoblotting, the samples were subjected to 7.5% or 10% SDS–PAGE, followed by blotting onto a polyvinylidene difluoride membrane (Millipore). Non-specific binding sites were blocked by incubation with 5% skim milk in 0.01 M PBS containing 0.03% TritonX-100 (PBS-T) at 4 °C overnight. The membrane was incubated with anti-GFP antibody for 1 h at room temp. After washing with PBS-T, the membrane was incubated with peroxidase-labeled anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 30 min. After three rinses with PBS-T, the immunoreactive bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech).

To determine kinase activity of DGKβ and mutants, appropriate volume of the homogenate samples, containing comparative amounts of the fusion protein of DGKβ or mutants, were subjected to octyl-glucoside-mixed-micelle assay [17]. 1-Steroyl-2-arachidonoyl-sn-glycerol (BIOMOL) was used as substrate. The radioactivity of PA was separated on 20 cm Silica gel 60 TLC plate (MERCK) using a chloroform:methanol:acetic acid (65:15:5) solution and detected by BAS2500 (Fujix).

2.2.4. Lipofection

SH-SY5Y cells (5.0×10^4 cells/dish) grown on a glass-bottom dish (MatTek Corp) were transfected using 3 μl of FuGENE™6 Transfection reagent (Roche Molecular Biochemicals) and 1 μg of DNA according to the manufacturer's protocol. Transfected cells were cultured at 37 °C for about 24 h before use.

2.2.5. Confocal laser scanning microscopy analysis

The fluorescence of the GFP was observed with a confocal laser scanning fluorescent microscope (LSM 510 invert, Carl Zeiss) at 488-nm argon excitation using a 515–535-nm band pass barrier filter. The image was analyzed with Neurolucida and Nurolucida Explorer software (MBF Bioscience, Tokyo, Japan) to count the number of neurites and branches.

2.2.6. Statistical analysis for induction ability of DGKβ mutants

More than 100 cells were observed in each experiment and categorized into three groups, (a) no neurites, (b) one or two long neurites without branches, and (c) more than three neurites with branches. The mean and SEM of the number of type (a), (b) or (c) cells in the three independent experiments are plotted. Probability between same type of cells expressing DGKβ, its mutants or GFP alone was compared by one-way ANOVA (followed by Tukey's test). Statistical significance is indicated by P values less than 0.001.

3. Results

3.1. Neurite induction ability of the DGKβ mutants

First, we examined the neurite induction ability of a series of domain deletion mutants of DGKβ (Fig. 1A). All mutants were the appropriate molecular weight without any significant degradation, although a small amount of degraded product was detected in the cases of CA and IncABS (Fig. 1B).

SH-SY5Y cells overexpressing GFP-fused full length DGKβ (DGKβFL) had more neurites and branches than control cells expressing GFP alone; statistical analysis showed that approximately 50% of the cells overexpressing DGKβFL had several neurites with branches, whereas this proportion was only 10% in the control cells expressing GFP (Fig. 2A). More than 50% of the cells expressing control GFP had no neurites; this proportion was only 20% in the DGKβFL cells. In contrast, the C-cut mutant lost its ability to induce neurites; more than 60% of the cells overexpressing GFP-DGKβ C-cut had no neurites (Fig. 2A). These results indicated that the 34 amino acids at the C-terminus of DGKβ (C-terminus) are important for neurite induction ability.

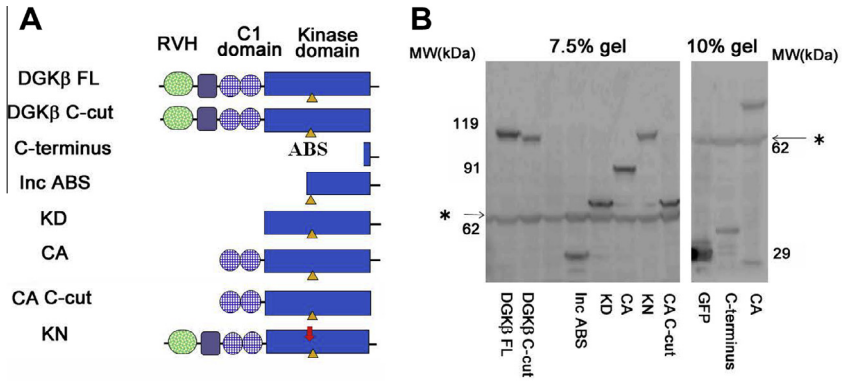

Fig. 1. Schematic illustration of DGKβ mutants and their molecular weights. (A) GFP was omitted from the schematic illustration, although all DGKβ mutants have GFP at the N-terminus. RVH, recoverin homology domain; FL, full length; ABS, putative actin binding site; KD, kinase domain; CA, constitutively active; KN, kinase negative. Red arrow represents a mutation in the ATP binding site. (B) Immunoblotting was performed using two types of SDS gels. The numbers at right and left sides are the molecular weights. * indicates non-specific bands. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

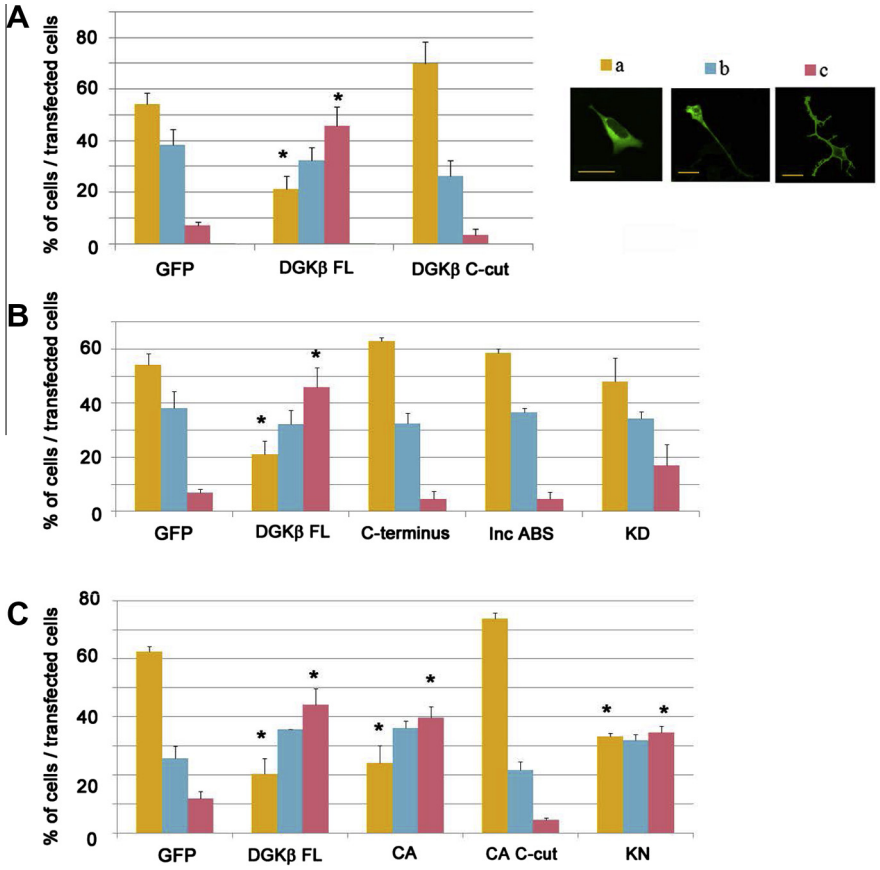

Fig. 2. Neurite induction ability of DGKβ mutants. (A) Importance of C-terminus for neurite induction. (B) Insufficiency of the kinase domain for neurite induction. (C) Importance of the C1 domain for neurite induction. In single experiment, more than 100 cells were categorized as having no neurites (a), one or two long neurites without branches (b), and more than three neurites with branches (c) as shown at the right side. The mean and SEM of the number of type (a), (b) and (c) cells in the three independent experiments are shown in orange, blue and pink column respectively, as a percentage of the transfected cells. * indicates a significant difference in the respective categories ($P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Therefore, we determined whether the C-terminus is sufficient to induce neurites and branches. When the GFP-fused the C-terminus was overexpressed in SH-SY5Y cells, the mutant did not induce neurite formation (Fig. 2B), suggesting that an additional domain is responsible for inducing neurites. Then, we extended the fusion protein to the middle of kinase domain (IncABS) because there is a putative actin binding motif (LEXXES) seen in PKCε and α-actinin [18] in the middle of kinase domain, and to the entire kinase domain (KD). Neither mutant had significant effects on neurite induction (Fig. 2B), although KD slightly induced neurites. When the C1 domain was further added to KD (CA), thereby forming a constitutively active form like DGKα [19], the neurites and branches were induced similarly to that of DGKβFL (Fig. 2C). Finally, to confirm the importance of the C-terminal region, we removed 34 amino acids from CA and investigated its neurite induction ability. As expected, CA C-cut lost its neurite induction ability (Fig. 2C). These results indicated that both the C-terminus and the C1 domain were necessary for neurite and branch induction ability.

3.2. Correlation between the neurite induction ability and kinase activity or localization of the DGKβ mutants

Next, we compared the kinase activity and localization of the mutants.

KD and CA showed approximately 50% and 300% kinase activity, respectively, compared with DGKβFL (Fig. 3A). The other mutants did not have any significant activity. To further investigate the correlation between kinase activity and neurite induction ability, we produced a kinase negative mutant (KN) that has a Gly-to-Asp substitution at position 495 in the ATP binding site. KN lost kinase activity (Fig. 3A) but was able to induce neurites, although the rate of induction was less than that of WT and CA (Fig. 2C). These results indicated that kinase activity does not fully account for their neurite induction ability and suggests other important factors are necessary for neurite induction.

Therefore, we focused on the localization of the DGKβ mutants. As shown in Fig. 3B, DGKβFL was mainly localized on the plasma membrane in SH-SY5Y cells as well as CA and KN. In contrast, C-terminus and IncABS were localized in the nucleus, and DGKβC-cut and KD were expressed in the cytoplasm. CA C-cut was localized both in the nucleus and cytoplasm. The localization patterns of these mutants were confirmed in CHO-K1 cells (data not shown). In other words, all mutants that induced neurites and branching showed clear plasma membrane localization similar to DGKβFL, whereas the other mutants that lost plasma membrane localization did not induce neurites. These results suggested that plasma membrane localization via the C1 domain and the C-terminus is critical for the neurite- and branch-induction ability of DGKβ.

3.3. Identification of important amino acids in the C1 domain and C-terminus for neurite induction ability and membrane localization

The C1 domain consists of two cysteine-rich regions, C1A and C1B. To determine which domain is important for neurite induction ability, we produced a C1A and a C1B mutant. The former and the latter have a Cys-to-Gly substitution at the third Cys in the C1A or C1B domain (Fig. 4A), based on the report that this mutation affects the entire structure of the domain of PKC [20]. Both mutants were localized in the cytoplasm in SH-SY5Y cells (Fig. 4B) and lost their neurite induction ability (Fig. 4C), indicating that the entire structure of the C1 domain is important for the neurite induction ability and plasma membrane localization of DGKβ.

Finally, to determine the amino acids responsible for neurite induction and membrane localization at the C-terminus, we constructed several mutants as described in Fig. 4D. We focused on Pro–Pro–Pro because proline-rich domains are often involved in protein–protein binding [21]. However, mutation of the three prolines (PPP/AAA) did not affect plasma membrane localization or neurite induction ability (Fig. 4E). Then, we put attention on Ser 795 and Thr 799 because these amino acids are assumed to be phosphorylated by PKC [22], and DGKγ has PKCγ phosphorylation site(s) at the C-terminus [23]. However, the DGKβ ST/A mutant, which has Ser-to-Ala and Thr-to-Ala substitutions, still showed significant plasma membrane localization and neurite induction ability (Fig. 4E and F). Finally, we focused on a cluster of basic amino acids located at the very end of the C-terminus (Lys–Arg–Thr–Arg–Asn–Arg–Ser–Lys–Glu, amino acids 793–801). Among the basic amino acids, Arg at 794, Arg at 796, and Lys at 800 were substituted to Glu (KR/E mutant). KR/E mutant lost membrane localization and neurite induction ability (Fig. 4E and F). These results indicated that electric charge produced by basic amino acids was involved in the plasma membrane localization and neurite induction ability of DGKβ.

4. Discussion

Similar to other type I DGKs, DGKα and γ, DGKβ consists of several domains, including the RVH domain, EF-hand domain, C1 domain, and kinase domain. The role of each domain in DGKβ has not been fully elucidated. Here, we obtained novel information regarding the roles of at least three regions of DGKβ on activity, localization, and neurite induction ability.

The N-terminus region, including the RVH and EF-hand domains, is thought to be a calcium ion sensor. Indeed, the kinase activity of DGKα is calcium dependent, and the RVH and EF-hand domains are necessary for calcium sensitivity [19]. In addition, the N-terminus region of DGKα also has an inhibitory effect on kinase activity [19,24]. The present study revealed that the RVH and

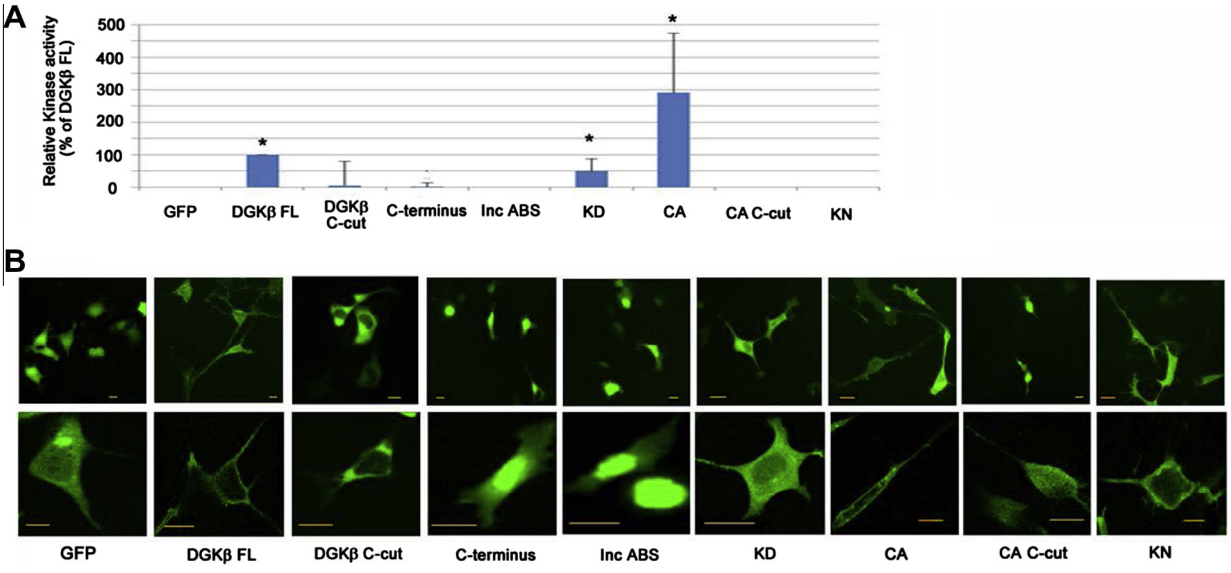

Fig. 3. Characteristics of DGKβ mutants. (A) Relative kinase activity of DGKβ mutants. A kinase assay was performed as described in the experimental procedures, and the results are plotted as activity relative to DGKβ FL. Each bar shows the average of three independent experiments with standard error. * indicates a significant difference compared to the control GFP ($P < 0.05$) by student's *t*-test for ANOVA analysis. (B) Localization of DGKβ mutants in SHSY-5Y cells. Lower panel shows magnified images of the cells showing typical localization of the mutant were chosen. Bars = 10 μm.

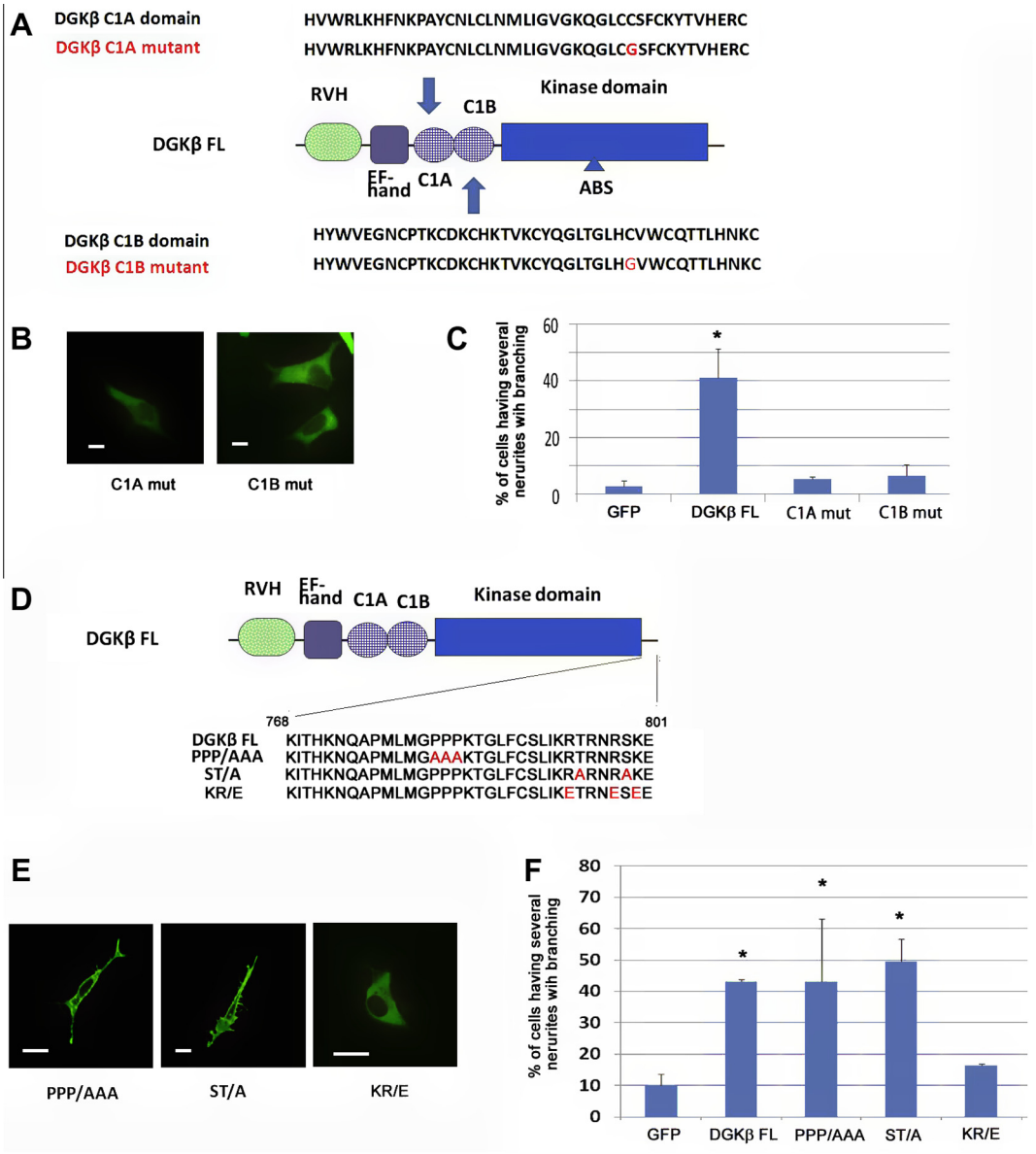

Fig. 4. Identification of amino acids important for neurite-induction in C1 domain and C-terminus. (A) Schematic illustration of C1A or C1B mutants. Third Cys in the C1A or C1B domain was substituted with Gly as shown in red. (B) Typical localization of C1A or C1B mutants in SH-SY5Y cells. Bars = 10 μm. (C) Importance of entire C1 domain for neurite induction. (D) Schematic illustration of PPP/AAA, ST/A, and KR/E mutants. The substituted amino acids are shown in red. (E) Typical localization of PPP/AAA, ST/A, and KR/E mutants in SH-SY5Y cells. (F) Importance of a cluster of basic amino acids at C-terminus for neurite induction. (C, F) Only the mean and SEM of the number of cells with more than three neurites with branches are shown as a percentage of the transfected cells. *Indicates significant differences ($P < 0.001$). Bars = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

EF-hand regions of DGKβ also inhibit kinase activity, although the calcium dependence of DGKβ activity has not been shown. These results suggest that the N-terminus region of type I DGK masks the kinase domain to maintain it in an inactive state. Indeed, in the case of DGKα, the N-terminus region directly binds to its constitutively active form in a calcium-dependent manner [25].

We showed that C1 domain of DGKβ was indispensable for the plasma membrane localization and kinase activity. This was supported by the report that the C1 domain of DGKα was also important for the plasma membrane localization [26]. However, unlike in DGKβ, deletion of the C1 domain from the constitutively active form of DGKα did not affect the kinase activity [26]. This result may be due to the differences between the C1 domains of DGKα and β; the C1 domain of DGKβ, but not that of DGKα, can bind phorbol ester [27].

The C-terminus region of DGKβ was necessary for the plasma membrane localization and kinase activity. Similarly, a splice variant of human DGKβ, DGKβ SV3, showed cytosolic localization [14] and truncation of the C-terminus of a constitutively active form of DGKα abolished the plasma membrane localization [26]. Moreover, the C-terminus of DGKβ was important for kinase activity. In contrast, Caricasole et al. reported that DGKβ SV3 possesses significant kinase activity [14]. These disparate results may be due to the difference between the DGKβ SV3 and C-cut. We removed 34 amino acids to construct the C-cut mutant, whereas there are an additional 4 amino acids after the 35-amino-acid deletion in SV3. This slight difference between C-cut and SV3 may account for the discrepancy. It is noteworthy that the importance of C-terminus for kinase activity is supported by previous result that fusion of GFP to the C-terminus of DGKγ abolished its kinase activity [28].

Most importantly, we showed for the first time that both the C-terminus and the C1 domain are critical for the neurite and branch induction ability and plasma membrane localization of DGKβ. The lipid binding ability of the C-terminus may be important for DGKβ localization on the plasma membrane and neurite formation because a cluster of basic amino acids of some proteins is known to bind to acidic phospholipids on the plasma membrane [29]. The C1 domain of DGKβ also has lipid binding ability [27]. The lipid binding ability of the C-terminus and/or C1 domain enables DGKβ to localize on the plasma membrane, thereby affecting the composition and structure of the membrane lipids and leading to changes in shape [29]. The C1 domain is also known to interact with some proteins [30,31]; thus, binding partners may also be important. The binding proteins to the C1 domain may regulate the actin cytoskeleton and/or membrane to induce neurite formation.

Our hypothesis is supported by several lines of evidence. First, PKCε also kinase-independently induces neurites through plasma membrane localization via PIP_2 and actin binding, although PKCε induces one or two long neurites without any branches [32–34]. In addition, PKCη can regulate the shape of keratinocytes in a kinase-independent manner [31]. In this case, the plasma membrane localization via the C1 domain was critical, similarly to what was observed with DGKβ and PKCε. We have recently identified RalA, a small GTPase, as a binding protein to the C1 domain of PKCη, which is involved in PKCη-induced morphological changes. Moreover, RalA has been reported to induce filopodia [35]. Accordingly, RalA is one of candidates that regulate DGKβ-induced neurites and branches, although further experiments are necessary.

In conclusion, this study revealed that the plasma membrane localization of DGKβ via the C1 domain and the C-terminus is necessary for its neurite and branch induction ability. Specifically, the importance of a cluster of basic amino acid is intriguing for many researchers because the region is associated with an isoform found in patients with bipolar disorder.

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.2014.03.113.

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