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Copine1 C2 domains have a critical calcium-independent role in the neuronal differentiation of hippocampal progenitor HiB5 cells

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ABSTRACT

Copine1 (CPNE1) has tandem C2 domains and an A domain and is known as a calcium-dependent membrane-binding protein that regulates signal transduction and membrane trafficking. We previously demonstrated that CPNE1 directly induces neuronal differentiation via Akt phosphorylation in the hippocampal progenitor cell line, HiB5. To determine which region of CPNE1 is related to HiB5 cell neurite outgrowth, we constructed several mutants. Our results show that over-expression of each C2 domain of CPNE1 increased neurite outgrowth and expression of the neuronal marker protein neurofilament (NF). Even though protein localization of the calcium binding-deficient mutant of CPNE1 was not affected by ionomycin, this mutant increased neurite outgrowth and NF expression in HiB5 cells. Furthermore, Akt phosphorylation was increased by over-expression of the calcium binding-deficient CPNE1 mutant. These results suggest that neither cellular calcium levels nor the localization of CPNE1 affect its function in neuronal differentiation. Collectively, our findings indicating that the C2 domains of CPNE1 play a calcium-independent role in regulating the neuronal differentiation of HiB5 cells.

1. Introduction

Copines are a family of calcium-dependent lipid-binding proteins that are evolutionally conserved from *Arabidopsis* to *Homo sapiens*. Nine human copine family members have been identified [1,2]. Copine1 (CPNE1) is soluble, calcium-dependent membrane-binding protein found in a variety of organisms [3]. It is ubiquitously expressed in various tissues and organs, including the brain [4]. Copine1 has two tandem C2 domains (C2A and C2B) at the N-terminus, and an A domain at the C-terminus [4].

The A domain is named from von Willebrand Factor, a plasma and extracellular matrix protein [5]. A domains have been studied in integrins and several extracellular matrix proteins and appear to function as protein-binding domains [5]. It was previously reported that copine1 binds with various intracellular proteins via its A domain [6,7].

The C2 domain is a calcium-dependent phospholipid-binding motif that was originally identified in protein kinase C [8]. Most proteins containing C2 domains are involved in signaling pathways and membrane trafficking, such as protein kinases, lipid kinases, phospholipases, and GTPase-activating and membrane trafficking proteins like synaptotagmin, rabphilin, and DOC2 [8,9]. The C2 domains of copines contain aspartate residues important for calcium and phospholipid binding [8,10]. C2 domains of CPNE1 exhibit Ca^{2+}-dependent phospholipid binding activity, which could affect the subcellular localization of copine proteins in response to Ca^{2+} stimuli [11]. Accordingly, the C2 domains of CPNE1 may be involved in cell signaling and/or membrane trafficking pathways [3,11]. However, the role of CPNE1 in regulating biological processes is not well understood.

Akt is involved in a number of biological functions including cell proliferation, differentiation, and survival [12]. Recently, Akt was reported to be a key mediator of neurite outgrowth and also promotes neuronal survival [13]. CPNE1 phosphorylates Akt on serine residue 473 (S473) during HiB5 cell neuronal differentiation [14]. HiB5 cells are a hippocampal progenitor cell line derived from the hippocampal anlagen of embryonic day 16 rat embryos that was conditionally immortalized with a temperature-sensitive mutant allele of SV40 T antigen [15].

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In our previous study, we found that CPNE1 enhanced HiB5 neuronal differentiation not through the phosphoinositide 3-kinase (PI3K)-Akt pathway but through another Akt activated signaling pathway [14]. Here, we provide the first evidence that neuronal differentiation of HiB5 cells induced by CPNE1 is related to its C2 domains and not its A domain. Furthermore, this differentiation is independent of calcium concentration and CPNE1 subcellular localization.

2. Materials and methods

2.1. Plasmid constructs and mutagenesis

Full-length CPNE1 cDNA (GenBank Accession No. BC001142) was obtained from KRIBB (Korea Research Institute of Bioscience and Biotechnology), Daejeon, Korea. The full-length clone was generated using a cDNA template, via a polymerase chain reaction (PCR)-based gateway cloning method as described previously [16]. C2A domain, C2B domain, and A domain mutants were obtained by using full-length cDNA as a PCR template. A site-directed mutagenesis system (Enzynomics) was used to construct of Ca^{2+}-binding-deficient mutant forms of CPNE1 (C2A-D21A, C2A-D90A, C2A-D21A/D90A, C2B-D216A, C2B-D222A, C2B-D216A/D222A, CPNE1-$C2A_{cbm}$ (D21A/D90A), CPNE1-$C2B_{cbm}$ (D216A/D222A), CPNE1-$C2A_{cbm}C2B_{cbm}$ (D21A/D90A/D216A/D222A). The resulting PCR products were cloned into destination vectors pDEST-AD-GFP using the gateway cloning system (Invitrogen).

2.2. Cell culture

HEK293A cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Immortalized HiB5 cells, a rat neuronal stem cell line, were cultured in DMEM supplemented with 1% penicillin–streptomycin and 10% FBS. Cells were maintained at 33 °C with 5% CO_2.

2.3. Adenovirus amplification and infection

Adenovirus was prepared and propagated in HEK293A cells using the ViraPower™ Adenoviral Expression System (Invitrogen). pDEST-AD-GFP-tagged gene expression vectors were transfected into HEK293A cells to obtain adenovirus particles. After 7–10 days, virus particles were harvested from the cells and media, and then purified by centrifugation at 3000 rpm for 15 min. For adenoviral infection, HiB5 cells were plated in 6-well plates at a density of 1×10^5 cells/mL and infected with adenoviruses at a multiplicity of infection (MOI) of 100 and were then incubated for 72 h at 33 °C.

2.4. Imaging analysis and neurite outgrowth quantification

Several randomly chosen fields of cells infected with Ad-GFP and Ad-GFP-tagged constructs were photographed using a confocal microscope (Olympus Fluoview FV-1000), and cells with processes longer than double the cell body diameter were scored as neurite outgrowth cells. Aggregated cells were not scored, nor were cells bearing atypical processes, such as hair-like branched processes. The data are expressed as a percentage of neurite outgrowth cells out of the total number of GFP-expressing cells.

2.5. Immunoblot analysis

HiB5 cells were lysed with RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM EDTA, 1 mM PMSF, and 1% NP-40) containing a protease-inhibitor cocktail. Whole-cell lysates were incubated on ice for 30 min and then cleared at 20,000×*g* for 20 min at 4 °C. Cell lysates were separated by SDS–PAGE using 10% gels. The separated proteins were transferred to PVDF (polyvinylidene fluoride) membranes. The blots were probed with anti-neurofilament (NF) (1:1000, Covance), anti-CPNE1 (1:1000, Santa Cruz Biotechnology), anti-α-tubulin (1:2000, Sigma–Aldrich), anti-AKT1/2/3 (1:1000, Santa Cruz Biotechnology), and anti-phospho-AKT1/2/3 (S473; 1:1000, Santa Cruz Biotechnology) antibodies. Blots were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies, followed by washing and immunoreactivity detection with enhanced chemiluminescence (ECL, Abfrontier).

2.6. Live cell imaging

COS-7 cells were cultured in DMEM supplemented with 1% penicillin–streptomycin and 10% FBS. Cells were maintained 37 °C with 5% CO_2. Before transfection, cells were plated onto glass coverslips coated with poly-D-lysine. When the cells were 70–80% confluent, they were transfected with GFP-tagged gene expression vectors using Lipofectamine 2000 reagent (Life Technologies). Cells were imaged 24 h after transfection. The coverslips were transferred to a slide-holder chamber containing 0.4 mL imaging buffer (150 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 2 mM $CaCl_2$, 5.3 mM glucose, and 10 mM HEPES-NaOH [pH 7.4]) and treated with 5 μM ionomycin during visualization with a Olympus FV1000 confocal microscope for live cells.

2.7. Statistical analysis

Statistical analyses were performed with Origin 8.0 software using analyses of variance (ANOVAs). Statistical significance was set at $P < 0.05$. Data are reported as the means ± standard deviations (SDs) of three independent experiments.

3. Results

3.1. Effect of truncated CPNE1 mutants on neuronal progenitor HiB5 cell differentiation

We previously reported that CPNE1 increases Akt phosphorylation, neuronal marker protein (Tuj1) levels, and neurite outgrowth in HiB5 cells, indicating neuronal differentiation [14]. To determine which region of CPNE1 is related with neuronal differentiation, we constructed adenoviruses containing GFP-CPNE1, -C2A, -C2B, and -A domain mutants (Fig. 1A). These truncated mutants were expressed in HiB5 cells, a model cell line for neuronal differentiation, and we measured neurite outgrowth and morphological changes (Fig. 1B). When C2A, C2B, and wild-type CPNE1 were over-expressed in HiB5 cells, the number of neurite outgrowth cells increased approximately 5 times compared with GFP-control-infected cells after a 72-h incubation (Fig. 1B and C). However, neurite outgrowth was barely altered in A domain-infected cells (Fig. 1B and C).

To reconfirm the role of these truncated mutants in neuronal differentiation, we assessed expression of the neuronal marker NF and Akt (S473) phosphorylation by infecting HiB5 cells with these mutants (Fig. 1D and E). As expected, NF expression was increased approximately 3–4-fold following the over-expression of C2A, C2B, and wild-type CPNE1 but after A domain mutant infection (Fig. 1D). Furthermore, phospho-Akt (S473) levels increased approximately 2-fold in the C2A, C2B, and wild-type CPNE1-infected cells compared with GFP only-infected cells at 72 h after infection (Fig. 1E). However, the level of phospho-Akt (S473) was

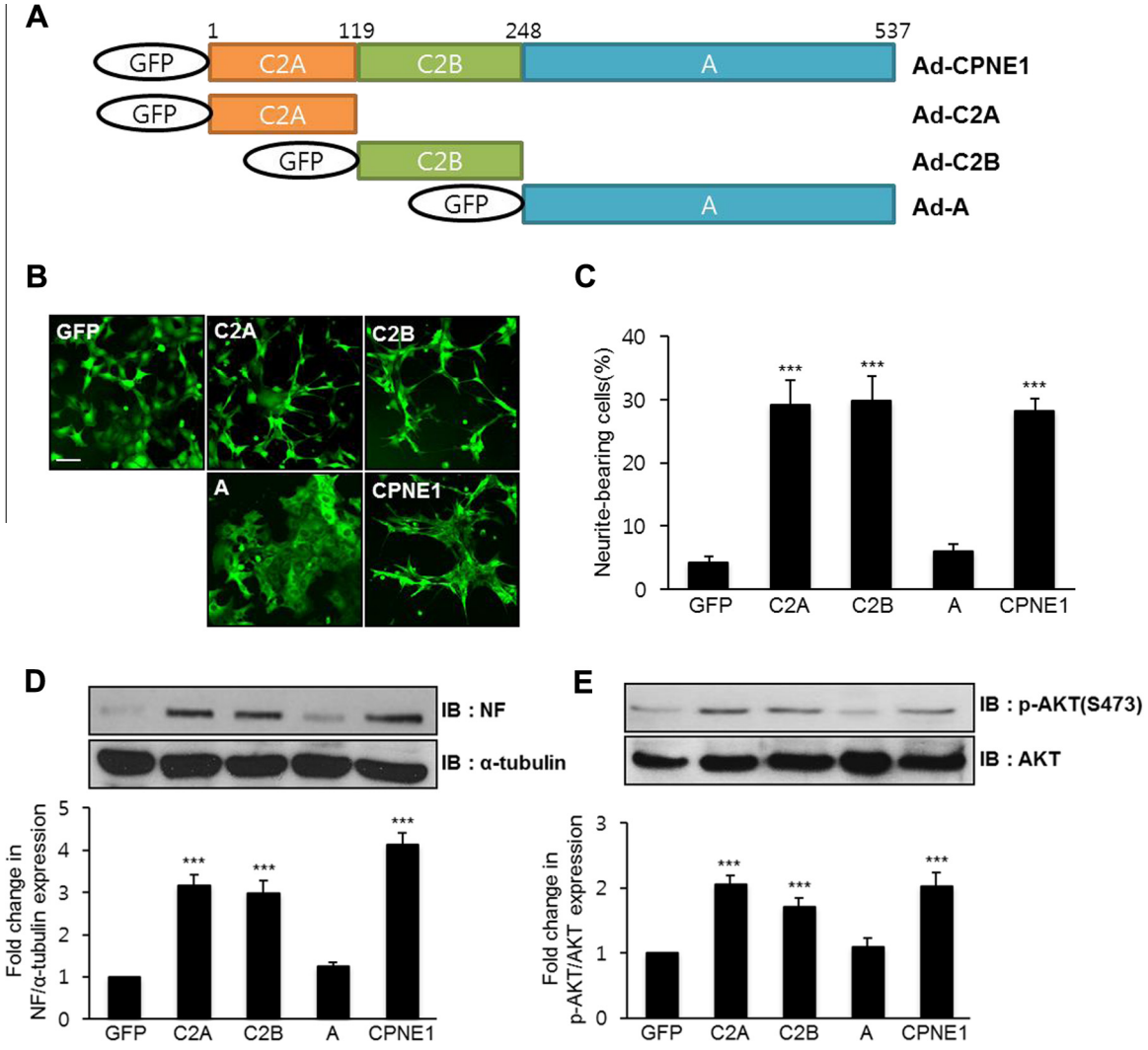

Fig. 1. Each C2 domain of CPNE1 has effects on HiB5 cell neurite outgrowth. (A) Schematic of the truncated CPNE1 mutants used in this experiment. (B) HiB5 cells were infected with the Ad-GFP-expressing constructs in (A) for 72 h, and cell morphology was imaged using confocal microscopy (Scale bar = 20 μm). (C) Bar graph showing the percentages of neurite outgrowth in GFP-expressing cells (***$P < 0.001$, ANOVA). Expression levels of the neuronal marker protein NF (D) and phospho-Akt (S473) (E) were analyzed 72 h after infection. The Bar graph shows the mean percentages of NF (D) and phosphor-Akt (S473) (E) from three independent experiments (***$P < 0.001$, ANOVA). α-Tubulin expression was used as a loading control.

almost same in the A domain-infected cells compared with GFP only-infected cells (Fig. 1D and E).

These results demonstrate that C2 domains but not the A domain of CPNE1 have a pivotal role in neuronal differentiation.

3.2. Effect of the D21A and/or D90A mutants of the C2A domain on neuronal differentiation

It is known that copine C2 domains are calcium-dependent lipid-binding domains [11]. To assess whether neuronal differentiation induced by the C2 domain was dependent on calcium ion (Ca^{2+}) levels, we constructed calcium-binding-deficient mutants for the C2A domain (D21A, D90A, and D21A/D90A) and C2B domain (D216A, D222A, and D216A/D222A) (Fig. 2A and Supplementary Fig. 1A). These mutants were used to infect HiB5 cells, and then we measured neurite outgrowth and morphological changes (Fig. 2B). When C2A, C2A-D21A, C2A-D90A, and C2A-D21A/D90A domains of CPNE1 were over-expressed in HiB5 cells, all of mutants exhibited a ~5-fold increase in neurite outgrowth compared with GFP-control-infected cells (Fig. 2B and C). Similar results were obtained in cells infected with C2B, C2B-D216A, C2BA-D222A, and C2B-D216A/D222A constructs (Supplementary Fig. 1B and C).

To reconfirm the role of these C2A mutants in neuronal differentiation, we measured NF expression and Akt (S473) phosphorylation in HiB5 cells (Fig. 2D and E). As expected, expression level of NF, neuronal marker protein, was increased approximately 3–4-fold in the all of C2A mutants of CPNE1 infected cells (Fig. 2D). Furthermore, phospho-Akt (S473) levels increased approximately 2–3-fold 72 h after infection in the all of C2A mutants compared with GFP-control-infected cells (Fig. 2E). Similarly, NF and phospho-Akt (S473) levels were increased in the C2B mutants of CPNE1-infected cells (Supplementary Fig. 1D and E).

These results indicated that the calcium-binding role of C2A and C2B domains is not associated with the C2 domains effects on neuronal differentiation.

3.3. Neuronal differentiation induced by CPNE1 is unrelated to Ca^{2+} levels or protein localization

Because the C2 domains are related to HiB5 cell neuronal differentiation, we decided to examine whether Ca^{2+} concentration and

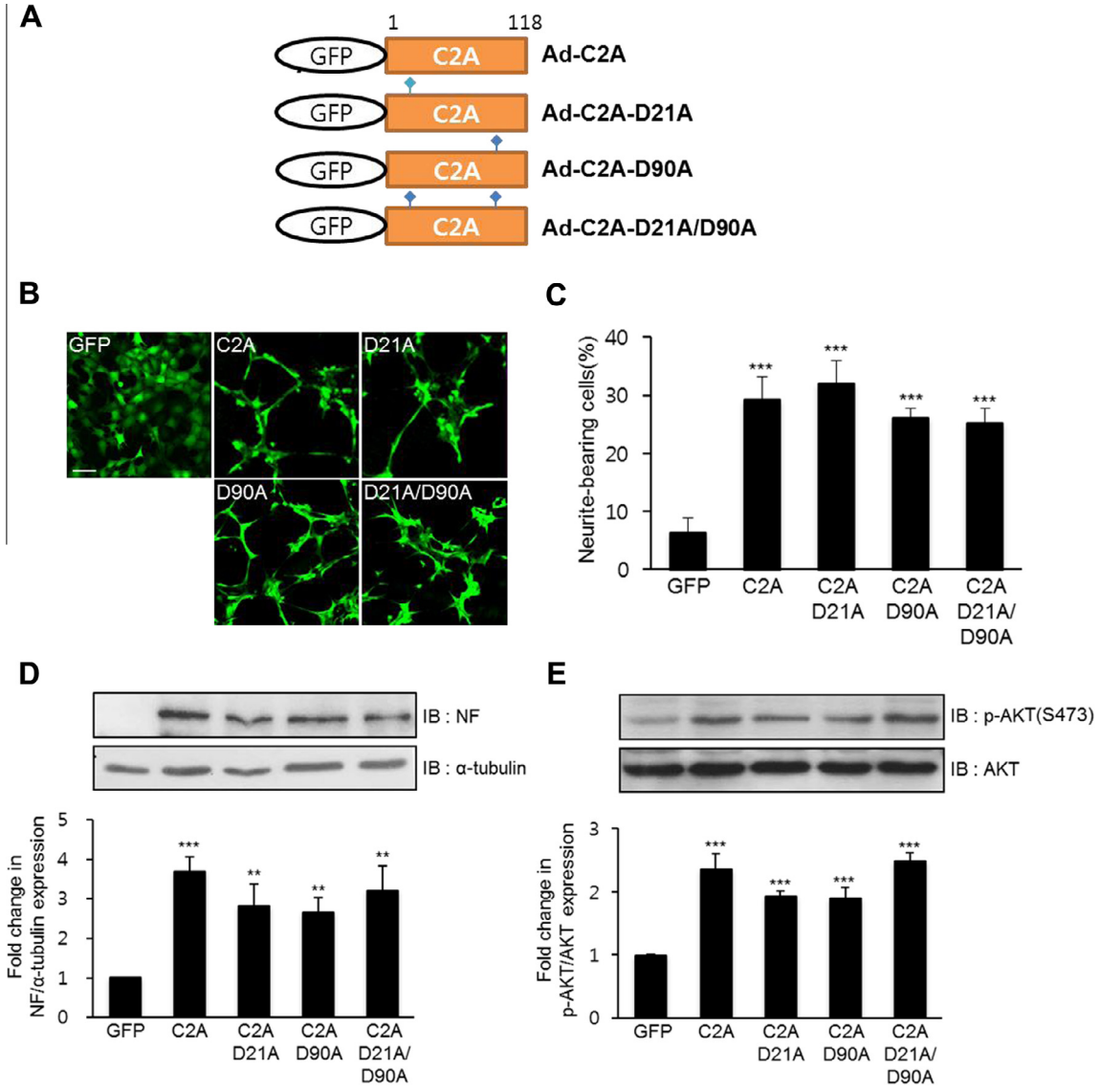

Fig. 2. Effect of Ca^{2+}-binding-deficient mutants of the C2A domain on CPNE1-mediatedd neuronal differentiation in HiB5 cells. (A) Schematic of the point mutants of the C2A domain of CPNE1 used in this experiment. (B) HiB5 cells were infected with Ad-GFP-expressing constructs in (A) for 72 h, and cell morphology was imaged using confocal microscopy (Scale bar = 20 μm). (C) The bar graph shows the percentages of neurite outgrowth in GFP-expressing cells (***$P < 0.001$, ANOVA). Expression levels of NF (D) and phospho-Akt (S473) (E) were analyzed 72 h after infection. The bar graph shows the mean percentages of NF (D) and phospho-Akt (S473) (E) from three independent experiments (***$P < 0.001$, **$P < 0.01$, ANOVA). α-Tubulin expression was used as a loading control.

CPNE1 localization are also involved in CPNE1-mediated neuronal differentiation. We constructed several calcium-binding-deficient mutants (CPNE1-$C2A_{cbm}$, CPNE1-$C2B_{cbm}$, and CPNE1-$C2A_{cbm}$ $C2B_{cbm}$) of wild-type CPNE1 (Fig. 3A). After these CPNE1 mutants were over-expressed in Cos7 cells, 5 μM ionomycin was added to the media (Fig. 3B). Ionomycin is an effective Ca^{2+} ionophore that is used to modify intracellular Ca^{2+} concentrations in cellular processes. After the addition of ionomycin, these CPNE1 mutants move into the membrane within 30 s (except CPNE1-$C2A_{cbm}$ $C2B_{cbm}$ mutants, Fig. 3B). This indicated that the localization shift of CPNE1 was Ca^{2+} dependent. Moreover, one of the calcium-binding regions of the C2A and C2B domains is necessary for movement toward the membrane in CPNE1. To confirm whether CPNE1 localization is related to neuronal differentiation, HiB5 cells were infected with these mutants. Then, we measured the levels of NF and Akt (S473) phosphorylation (Fig. 3C and D). Interestingly, NF expression was increased approximately 3–4-fold in the cells that over-expressed CPNE1 mutants (Fig. 3C). Furthermore, phospho-Akt (S473) levels increased approximately 2–3-fold in all of the mutant CPNE1-infected cells compared with GFP-control-infected cells at 72 h after infection (Fig. 3D). These results indicate that CPNE1 localization does not affect the effect of CPNE1 on neuronal differentiation in HiB5 cells. Collectively, our findings suggest that neuronal differentiation induced by CPNE1 is unrelated to Ca^{2+} levels and CPNE1 localization in HiB5 cells. This may indicate that the C2 domain has another regulating function that impacts neuronal differentiation.

4. Discussion

HiB5 cells are a multipotent hippocampal neuronal stem cell line [15]. Because the transfection efficiency is very low and it is difficult to assess the activity of transfected genes in these cells, we used recombinant adenoviruses containing GFP-control, GFP-CPNE1, and GFP-CPNE1 mutants to overcome this problem and measure the effect of CPNE1 on neuronal differentiation.

We previously reported a screening system for genes involved in neuronal differentiation and found that CPNE1 enhanced the

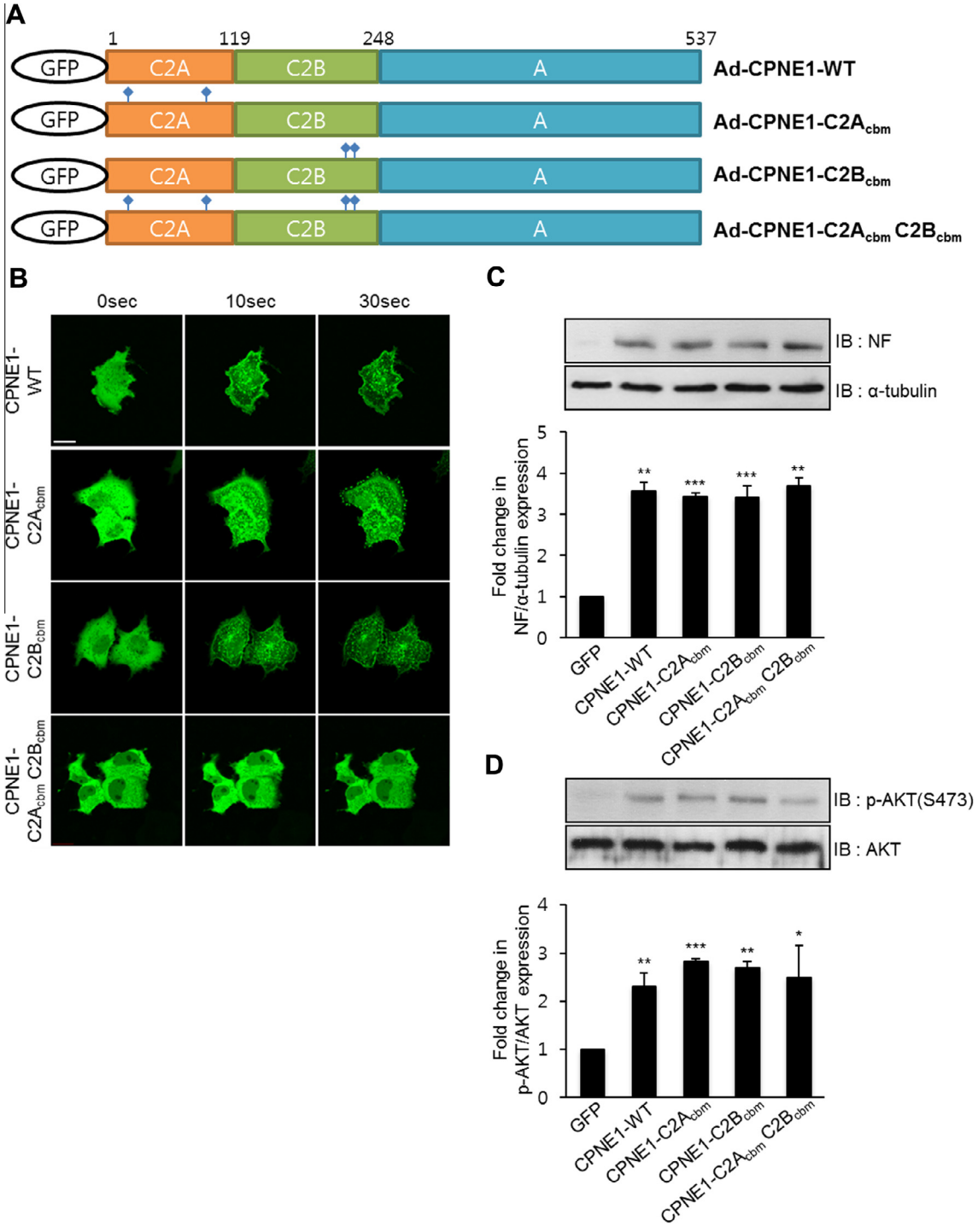

Fig. 3. Effect of Ca^{2+} and CPNE1 localization on HiB5 cell neuronal differentiation. (A) Schematic of the point mutants of CPNE1 used in this experiment. (B) Confocal images of the ionomycin responses of the Ca^{2+}-binding-deficient CPNE1 mutants in Cos7 cells (Scale bar = 20 μm). Expression levels of the neuronal marker protein NF (C) and phosphor-Akt (S473) (D) were analyzed 72 h after infection. The bar graph shows the mean percentages of NF (C) and phosphor-Akt (S473) (D) from three independent experiments ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, ANOVA). α-Tubulin expression was used as a loading control.

neuronal differentiation of HiB5 cells [14,16]. Therefore, we questioned which domain of CPNE1 was related with neuronal differentiation. Generally, C2 domains act as calcium sensors and direct the copine protein to the plasma membrane. The A domain is thought to bind copine target protein(s) [1]. Interestingly, each C2 domain not but the A domain of CPNE1 were found to affect neuronal differentiation in HiB5 cells (Fig. 1). This means that the C2 domain has another function in addition to its role as a calcium sensor.

Synaptotagmin possesses tandem C2 domains: C2A and C2B. Each C2 domain binds Ca^{2+}. Upon binding Ca^{2+}, positively charged residues within the Ca^{2+}-binding loops are thought to interact with negatively charged phospholipids in the target membrane [17]. By performing computer simulation and assessing the structure of the synaptotagmin C2 domain, we constructed calcium-binding-deficient mutants of C2A and C2B domains (Fig. 2 and Supplementary Fig. 1). The mutated C2 domains did not move into the plasma membrane following ionomycin treatment (data not shown).

However, these mutants had similar effects as wild-type CPNE1 C2 domains on neuronal differentiation (Fig. 2 and Supplementary Fig. 1). These results indicated that Ca^{2+} concentration is not related with the role of the C2 domain in neuronal differentiation.

It is known that calcium-binding-deficient mutation of one C2 domain of synaptotagmin affects the activity of the adjacent C2 domain [17]. To elucidate the exact function of C2 domains of CPNE1 in neuronal differentiation, we generated calcium-binding-deficient mutants of wild-type CPNE1 (Fig. 3A). Interestingly, each C2 mutant (CPNE1-C2A_{cbm} and CPNE1-C2B_{cbm}) but not the C2AC2B double calcium-binding-deficient mutant (CPNE1-C2A_{cbm} C2B_{cbm}) moved into the plasma membrane following ionomycin treatment (Fig. 3B). However, all of these mutants induced increased NF expression and Akt phosphorylation (Fig. 3C and D). Furthermore, all calcium-binding-deficient mutants had similar effects to wild-type CPNE1 on differentiation (Fig. 3B and C). Calcium-binding-deficient mutation of one C2 domain of synaptotagmin affects the activity of the adjacent C2 domain [17]. However, calcium-binding-deficient mutations of all C2 domains in CPNE1 are necessary to affect localization shifts induced by Ca^{2+} (Fig. 3B). These findings indicate that Ca^{2+} and CPNE1 localization barely affect CPNE1-mediated neuronal differentiation.

Over 20 proteins have been identified as copine targets, and a number of these are proteins involved in intracellular signaling pathways, such as MEK1/ERK, protein phosphatase 5, the CDC42-regulated protein kinase, and the NEDD8-conjugating enzyme UBC12 (ubiquitin C12) [6,7]. Moreover, it is known that copines are able to recruit these target proteins to phospholipid surfaces in response to changes in intracellular Ca^{2+} levels [11]. We found that CPNE1 regulates neuronal differentiation, but that this role is not affected by intracellular Ca^{2+} levels or CPNE1 localization (Fig. 3). In addition, CPNE1 membrane localization is possible even if one of the two C2 domains is mutated (Fig. 3B). This led to the hypothesis that CPNE1 may be able to induce neuronal differentiation through an Akt-specific cell signaling mechanism that does not require Ca^{2+} level changes [14]. Furthermore, these results may mean that the C2 domain itself has another function. It was recently reported that the C2 domain of PKCθ plays a major role in PKCθ activation via its Tyr(P) binding [18]. We are trying to find a possible upstream Akt activator, whose activity can be regulated by the C2 domain of CPNE1.

Here, we demonstrated that the C2 domains of CPNE1 have a major role in increasing neurite outgrowth in HiB5 cells. Furthermore, these events are not strongly associated with intracellular Ca^{2+} levels or and CPNE1 localization.

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.2014.10.075.

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