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14-3-3γ regulates Copine1-mediated neuronal differentiation in HiB5 hippocampal progenitor cells

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ABSTRACT

Copine1 (CPNE1), known as a calcium-dependent membrane-binding protein, has tandem C2 domains and an A domain. We previously demonstrated that CPNE1 directly induces neuronal differentiation via Protein kinase B (AKT) phosphorylation in the hippocampal progenitor cell line, HiB5. To better understand its cellular function, we carried out a yeast two-hybrid screening to find CPNE1 binding partners. Among the identified proteins, 14-3-3γ appears to directly interact with CPNE1. Between CPNE1 and 14-3-3γ, the physical interaction as well as the specific binding regions of CPNE1 was confirmed in vitro and in vivo. Furthermore, among the seven 14-3-3 isotypes, only 14-3-3γ directly interacts with CPNE1. Our results also demonstrate that AKT phosphorylation, neurite outgrowth and expression of the neuronal marker protein are increased when 14-3-3γ is overexpressed in CPNE1 high expressed HiB5 cells. Furthermore, the neighboring Ser54 amino acids residue of C2A domain in CPNE1 has an important role in binding with 14-3-3γ, and in differentiation-related function of CPNE1. Moreover, mutation of Ser54 amino acids residue in CPNE1 effectively decreased association with 14-3-3γ and neuronal differentiation of HiB5 cells. Collectively, our findings indicate that 14-3-3γ regulates the differentiation ability of CPNE1 through the binding with C2A domain of CPNE1 in HiB5 cells.

1. Introduction

Copine (CPNE) proteins are a family of ubiquitous calcium-dependent, phospholipid-binding proteins and evolutionary conserved from plants to humans [1]. In human, nine CPNEs (CPNE1-9) have been identified [2]. Most CPNE proteins are ubiquitously expressed, while CPNE6 shows brain-specific expression [3]. Some CPNE proteins have been shown to translocate to plasma membranes on calcium influx in heterologous expression system [4]. Recent studies reported that CPNE1 induces neuronal differentiation via AKT phosphorylation and CPNE6 regulates spine structural plasticity for learning and memory [5,6]. However, the functions of CPNEs in the brain are largely unknown.

Like other CPNEs, CPNE1 contains two N-terminal C2 domains (C2A and C2B), known to bind phospholipids in a calcium-dependent manner, and a C-terminal A domain (von Willebrand factor A domain) [4]. CPNE1 exhibit calcium-dependent phospholipid binding activities via its C2 domains and the subcellular localization of CPNE1 proteins can be affected in response to calcium stimuli [4]. CPNE1 binds with various intracellular proteins via its A domain [7]. CPNE1 induces phosphorylation of AKT and its C2 domains has a calcium-independent role during HiB5 cell neuronal differentiation [5,8]. HiB5 cells are a hippocampal progenitor cell line derived from the hippocampal anlagen of embryonic day 16 rat embryos. It was conditionally immortalized with a temperature-sensitive mutant allele of SV40 T antigen and easy to be handled in neuronal differentiation [9].

Abbreviations: CPNE1, Copine1; NF, neurofilament; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, Protein kinase B (PKB);; BiFC, bimolecular fluorescence complementation; VN/VC, N-terminal half/C-terminal half of Venus; IP, immunoprecipitation; MBP, myelin basic protein; GFP, green fluorescent protein; FBS, fetal bovine serum;; DMEM, Dulbecco's modified Eagle's medium; GBK, GAL4 DNA binding domain; GAD, GAL4 DNA activation domain; TL-, tryptophan/leucine lacking SD synthetic media; TLH-, trytophan/leucine/histidine lacking SD synthetic media

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14-3-3 family was known as adaptor proteins that can function by binding with their target proteins and inducing conformational changes [10]. Binding proteins of 14-3-3 proteins require three or four specific amino acids including phosphorylation of serine or threonine residues in the target sequence. The binding ability of 14-3-3 is regulated by forming homo or heterodimers among seven member of this family [10]. The highest concentration of 14-3-3 protein was found in brain tissue (about 1% of total soluble brain protein) [11]. 14-3-3 proteins are involved in a wide variety of cellular and physiological processes, including neuronal development, proliferation, and signal transduction via binding with a multitude of diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors [12]. Furthermore, 14-3-3 proteins were involved in neurodegenerative diseases such as Alzheimer disease, Parkinson's disease, and Creutzfeldt-Jakob disease [13].

In the present study, we have identified 14-3-3γ as an interacting protein of CPNE1 and confirmed that CPNE1 binds to 14-3-3γ in vitro and in vivo. Moreover, we found that Ser54 within C2A domain in CPNE1 is important for 14-3-3γ binding. Furthermore, we also provided data supporting the ability of 14-3-3γ regulating CPNE1-mediated differentiation with AKT protein activity as in hippocampal progenitor HiB5 cells. To our knowledge, this is the first time CPNE1/14-3-3γ binding and its differentiation effects have been investigated. These findings provide novel insights for understanding characteristics of CPNE1-related differentiation.

2. Materials and methods

2.1. Plasmid and shRNA construction

Full-length of CPNE1 cDNA (GenBank Accession No. BC001142) was obtained from Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejoen, Korea. The full-length clone was generated via a PCR-based gateway cloning method using cDNA as templates. C2A, C2B, A domain and C2A-deletion mutants were obtained by using full-length cDNA of CPNE1. CPNE1 (S54A) was obtained using a site-directed mutagenesis system (Enzynomics). Full-length 14-3-3γ cDNAs (GenBank Accession NM_012479.3) were generated using RT-PCR. The resulting PCR products were cloned into destination vectors pDEST-AD-GFP using the gateway cloning system (Invitrogen).

2.2. Cell culture

The HEK293A and COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin (PS) and 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. HiB5 cells were cultured in DMEM supplemented with 1% PS and 10% FBS at 33 °C or 39 °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 1000 rpm for 10 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 indicating time at 33 °C or 39 °C.

2.4. Yeast two-hybrid assay

The CPNE1 gene was cloned into pGBKT7, which encodes a GAL4 DNA binding domain (BD), while the 14-3-3γ gene was cloned into pGADT7, which encodes an activation domain (AD). To evaluate interactions of the two protein, both BD-CPNE1 and AD-14-3-3γ were co-transformed into the yeast strain AH109. The transformed yeast was then cultured in SD synthetic dropout medium lacking Trp, Leu and His (TLH-) for growth selection.

2.5. Immunocytochemistry and bimolecular fluorescence complement (BiFC)

HiB5 cells were grown on coverslips and were then fixed in 4% paraformaldehyde for 20 min.

Cells were permeabilized with 0.5% Triton X-100 in PBS and were blocked with 5% BSA in PBS. Cells were then incubated with the anti-CPNE1 (1:100, Santa Cruz Biotechnology) and anti-14-3-3γ (1:100, Abcam) antibodies for 1 h, cells were then washed and incubated with the Alexa fluor 488-conjugated secondary antibody and Alexa fluor 594-conjugated secondary antibody for 1 h. Cells were photographed using a confocal microscope (Olympus FluoView FV1000).

BiFC assays were evaluated after cloning full-length CPNE1 and 14-3-3γ genes into bimolecular fluorescence complement pBiFC-VN173 and pBiFC-VC155 vectors. COS7 cells were co-transfected with cloned pBiFC vectors in all possible pairwise combinations and were examined using imaging analyses as indicated above. Venus fluorescence signals were observed using confocal microscopy.

2.6. Co-immunoprecipitation and Immunoblot analysis

HEK293T cells and postnatal P1 mouse brain extract were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 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 13000 rpm for 30 min at 4 °C. For the immunoprecipitates analysis, the resulting supernatants were incubated with the indicated antibodies for 1 h at 4 °C with an additional incubation for 1 h after the addition of protein A/G plus agarose (Santa Cruz Biotechnology), which were then washed three times with RIPA buffer. For the western blot analysis, the immunoprecipitated proteins were separated on 10% SDS-PAGE gels and blotted onto PVDF membranes. The blots were then probed with anti-GFP, -CPNE1, -14-3-3γ and -GAPDH (1:1,000, Santa Cruz Biotechnology), anti-HA (1:1,000, Roche Applied Science), anti-FLAG and -α-tubulin (1:1,000, Sigma–Aldrich), anti-NF (1:1000, Covance), anti-AKT and -phosphor-AKT (Ser473) (1:1,000, Cell Signaling) antibodies. Blots were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies, followed by three washes and the detection with enhanced chemiluminescence (ECL; AbFrontier).

2.7. MBP pull-down assay

For MBP pull-down assays, MBP–14-3-3γ vector was transformed into the E. coli strain BL21. According to the manufacturer's protocol, MBP–14-3-3γ protein was purified (SpinClean; Mbiotech). FLAG-tagged CPNE1 was transfected into HEK293T cells, which were lysed after 24 h. The cell lysates were incubated with 1 μg of purified MBP–14-3-3γ on MBP Bind agarose resin (Elpis Biotech Inc., Korea) for 1 h at 4 °C. After three washes with lysis buffer, bound proteins were eluted by boiling with SDS sample buffer and subjected to western blot analysis.

2.8. In silico analysis of protein structure

PDB 2B05 was used for docking using LibDock in Discovery Studio (Biovia, San Diego CA). The proposed binding site was centered on the ligand and a site sphere was created (coordinates −46.344, −1.13216, −19.7663) with 11.23 Å diameter. The protocol included 100 hotspots and docking tolerance (0.25). The FAST conformation method was also