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Immunocytochemical Analysis of DGKη in Cultured Cells Using a Monoclonal Antibody DhMab-4

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Diacylglycerol kinase (DGK) is a lipid kinase that converts diacylglycerol (DG) to phosphatidic acid (PA). Since both DG and PA serve as intracellular second messenger molecules, DGK plays a pivotal role in balancing these two signaling pathways. Of the DGK family, DGKη is classified as a type II DGK. Reportedly, DGKη is expressed ubiquitously through mammalian tissues and cells. Previous studies using cDNA transfection methods reported cytoplasmic localization of DGKη in cultured human cells. However, subcellular localization of native protein is still unknown. Recently, we established a human DGKη-specific monoclonal antibody, DhMab-4. In this study, we examined subcellular localization of native protein of DGKη using DhMab-4 by immunocytochemistry in human cultured cells.

Keywords: DGKη, monoclonal antibody, immunocytochemical analysis

Introduction

Diacylglycerol (DG) is a unique lipid, which represents as an intermediate product of phospholipid metabolism and also serves as an intracellular signaling molecule. DG activates several proteins including conventional and novel types of protein kinase C (PKC), RasGRP, Unc-13, and canonical transient receptor potential channels.[1,2] DG is produced through several enzymatic pathways, such as (1) hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, (2) hydrolysis of phosphatidylcholine by sequential action of phospholipase D and phosphatidic acid (PA) phosphatase, and (3) acylation of monoacylglycerol (MG) by MG acyltransferase during triglyceride synthesis.[2–5]

Among DG-converting enzymes, diacylglycerol kinase (DGK) phosphorylates DG to produce PA.[1,3] Since PA, a product of DGK, also serves as a second messenger activating various proteins such as hypoxia inducible factor 1α, atypical PKCζ, and mammalian target of rapamycin, DGK is thought to balance DG-mediated and PA-mediated pathways.

DGK family consists of 10 isozymes in mammalian species.[1–3] Each isozyme has distinct molecular structures and subcellular localizations.[1,6] Of the DGKs, DGKη is classified as a type II DGK, which contains pleckstrin homology domain, two Zn -fingers (DG-binding C1 domain), separated catalytic domain, and sterile α-motif domain.[2,7] Reportedly, DGKη is expressed ubiquitously in mammalian organs, including the brain, liver, lung, kidney, thymus, spleen, and reproductive organ.[7–9]

Previous studies using cDNA transfection technique disclosed that DGKη localizes to the cytoplasm under normal conditions.[7,8] However, detailed localization of native DGKη protein remains undetermined, because no specific antibody is available for immunostaining. Herein, we report a novel anti-human DGKη (hDGKη) monoclonal antibody, DhMab-4, which is very useful in immunocytochemical analysis of cultured human cells.

Materials and Methods

Cell lines

HeLa and HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA), and were cultured in the Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA)

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at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Antibiotics, including 100 units/mL of penicillin, 100 μg/mL of streptomycin, were added to media.

Silencing RNA duplexes directed against hDGKη (siDGKη1, 5′- CCAAGACGCUAUGUGAAACUGUAAA-3′ and siDGKη2, 5′- CAAGGGAAAUCAUGUUGCGGGCAAA-3′) were purchased from Thermo Fisher Scientific, Inc. Scrambled siRNA duplexes (AllStars Negative Control siRNA; Qiagen, Valencia, CA) were used as control. HeLa cells were transfected with siRNAs against hDGKη or scrambled control using Lipofectamine RNAi MAX (Thermo Fisher Scientific, Inc.) according to manufacturer's instruction.

Immunocytochemical analysis

HeLa and HepG2 cells were fixed with 3% glyoxal fixative[10] for 30 minutes on ice, followed by at room temperature for additional 30 minutes. After fixation, cells were

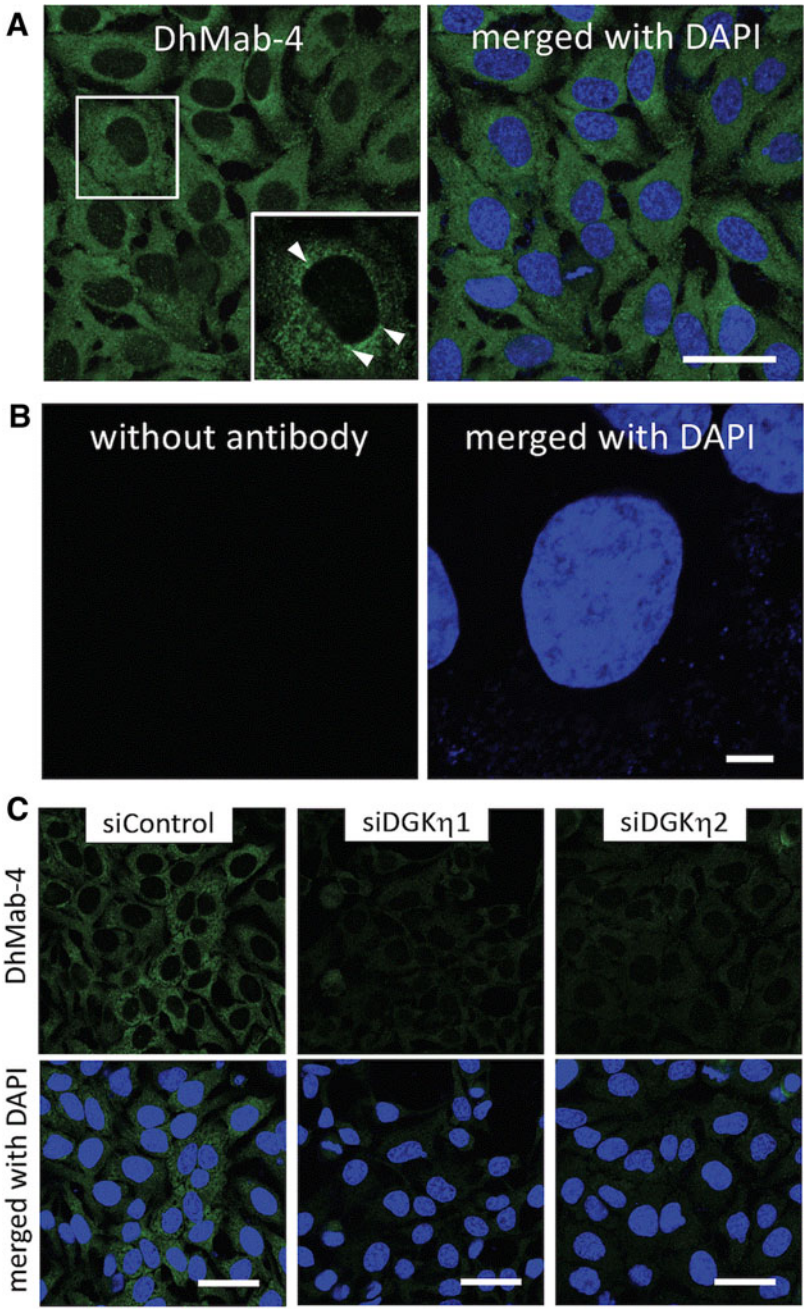

FIG. 1. Immunocytochemistry of HeLa cells using DhMab-4 in the presence **(A)** or absence **(B)** of the antibody. Inset in **(A)** is an enlarged view of squared area. Nuclear staining was performed with DAPI. Arrows indicate perinuclear staining. Scale bar = 10 μm. **(C)** Immunocytochemistry using DhMab-4 in siRNA-treated HeLa cells. Cells were transfected with siDGKη1 or siDGKη2 at an amount of 60 pmol/60 mm dish. After 48 hours, immunocytochemistry was performed using DhMab-4. Nuclear staining was performed with DAPI. AllStar Negative Control siRNA (siControl) was used as a control. Scale bar = 20 μm.

perforated with 0.3% Triton-X 100/phosphate-buffered saline (PBS) for 15 minutes at room temperature, followed by treatment with 5% normal goat serum (NGS) in PBS to block nonspecific binding sites, and were incubated with 0.5 μg/mL of DhMab-4 or control (NGS/PBS) for overnight at room temperature in a moist chamber. In some cases, cells were double stained with cytochrome oxidase subunit 4 (COX IV) (dilution 1:200; Cell Signaling Technology, Inc., Danvers, MA, USA), calreticulin (dilution 1:200; Novus Biological, Centennial, CO, USA) or Golgin 97 (dilution 1:200; Thermo Fisher Scientific, Inc.) antibodies as organelle markers.

Immunoreactions for DhMab-4 and organelle markers were visualized with anti-mouse IgG-Alexa 488 (dilution 1:300; Thermo Fisher Scientific, Inc.) and anti-rabbit IgG-Alexa 594 (Thermo Fisher Scientific, Inc.), respectively, for 30 minutes at room temperature. Cells were also reacted with DAPI (Thermo Fisher Scientific, Inc.) to stain the cell nuclei. They were examined using confocal laser-scanning microscopy (LSM700; Carl Zeiss, Inc., Jena, Germany).

Results and Discussion

Previous studies revealed that DGKη is targeted to the cytoplasm in transfected cultured cells including COS-7, HeLa, and NEC8 (human embryonal carcinoma cells derived from testis) cells.[7,8,11] In this study we aimed to investigate detailed subcellular localization of native DGKη protein in human cells. To this end, we established a specific monoclonal antibody against hDGKη (DhMab-4).[12]

In our previous study, DhMab-4 strongly stained Purkinje cells of human cerebellum in immunohistochemical analysis.[12] However, since it is hard to obtain fresh human brain specimen for morphological examination at subcellular level, we used in this study human cervical cancer cell line HeLa

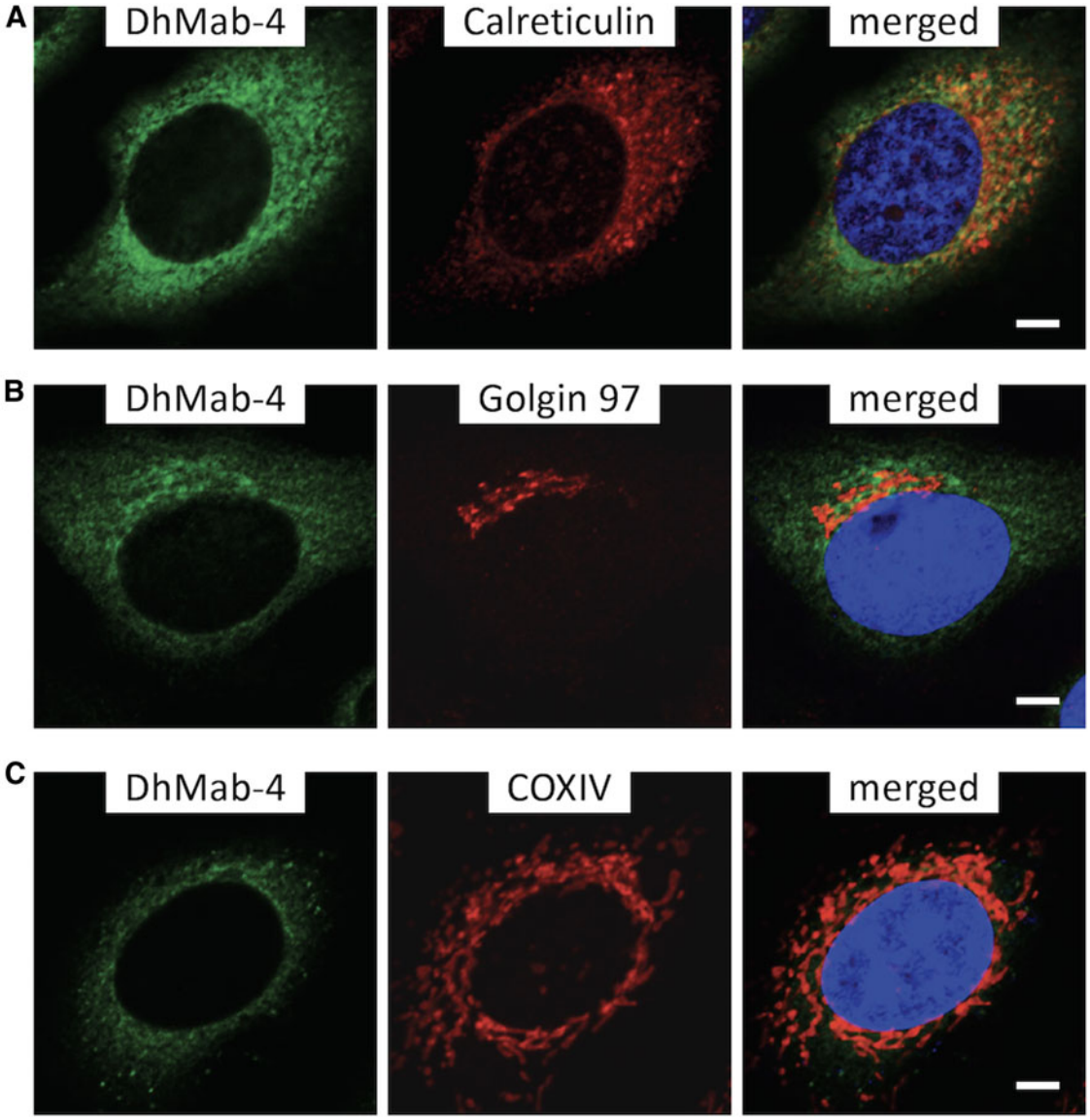

FIG. 2. Double immunostaining of DhMab-4 with the organelle markers in HeLa cells. **(A)** Anti-calreticulin antibody (endoplasmic reticulum), **(B)** anti-Golgin 97 antibody (trans-Golgi network), **(C)** COX IV antibody (mitochondria). Nuclear staining was performed with DAPI. Scale bar = 10 μm. COX IV, cytochrome oxidase subunit 4.

cells, in which DGKη protein expression is reported.[11] Immunocytochemical examination showed the immunoreactivity for DhMab-4 is predominantly detected in a granular manner in the cytoplasm (Fig. 1 A). On closer examination, the immunoreactivity was rather intensely recognized in the perinuclear region (arrows in Fig. 1A). The same staining pattern was obtained in another human cell line HepG2 cells (data not shown).

As a control experiment, immunostaining without primary antibody abolished the immunoreaction (Fig. 1B), showing no artifact in our antigen–antibody detection method. To verify the specificity of DhMab-4 in immunocytochemistry, we performed siRNA silencing experiment. As shown in Figure 1C, transfection with siRNAs for DGKη (siDGKη1 and -η2) significantly reduced DhMab-4 immunoreactivity, whereas transfection with scramble control siRNA (siControl) gave no effect. These results confirm that DhMab-4 stains native DGKη protein in human culture cells.

We next performed double immunostaining to determine whether native DGKη protein specifically localizes to some organelle in the cytoplasm. We used anti-calreticulin antibody (a marker for the endoplasmic reticulum[13]), anti-Golgin 97 (a marker for trans-Golgi network[14]), and anti-COX. IV (a marker for mitochondria). None of these marker antibodies was colocalized with DhMab-4 (Fig. 2). These double staining results demonstrate that DGKη native protein localizes to the cytoplasm but is not associated with distinct organelles such as endoplasmic reticulum (ER) Golgi complex, and mitochondria.

Reportedly, DGKη translocates to the endosomes by the osmotic stress in cDNA-transfection studies.[7,8] Considering our present data, granular pattern of DGKη protein in the cytoplasm might represent the endosomes, if not all. Endosomes are membrane-bound cytosolic vesicles, which include early, late, and recycling endosomal compartments. These small membrane-bound structures play some roles in endocytosis or cargo between the ER and Golgi complex.[15]

From the functional point of view, DGKη is identified as a bipolar disorder susceptibility gene.[16–18] Furthermore, DGKη is also implicated in oncogenic properties of lung cancers and DGKη knockdown leads to growth impairment of epidermal growth factor receptor pathway.[19] These results suggest that DGKη plays a pivotal role in higher brain function and tumorigenesis. Further study is needed to elucidate these points. This study showed that a novel anti-DGKη mAb, DhMab-4, specifically reacts with native hDGKη protein in immunocytochemistry and localizes to the cytoplasm of human cultured cells. To gain an insight understanding the relationship between these disorders and morphological aspects of DGKη, a specific antibody DhMab-4 would be a useful tool.

Author Disclosure Statement

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