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DzMab-1: Anti-Human Diacylglycerol Kinaseζ Monoclonal Antibody for Immunocytochemistry

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Diacylglycerol kinase (DGK) is an enzyme that converts diacylglycerol (DG) to phosphatidic acid (PA). As both DG and PA serve as lipidic second messengers, DGK plays a pivotal role in controlling the balance of two signaling pathways mediated by DG and PA in cellular functions. DGKζ, one member of the mammalian DGK family, is reported to contain a nuclear localization signal, which suggests its functional role in the nucleus. Previously, morphological studies using tagged expression vectors and immunostaining of rat tissues or cells have revealed that DGKζ localizes mainly to the nucleus. However, a limited number of studies reported the detailed localization of native protein of DGKζ in human tissues and cells. In this study, we developed a novel anti-human DGKζ monoclonal antibody, DzMab-1, which is very advantageous in immunocytochemistry of human cultured cells.

Keywords: DGKζ, monoclonal antibody, immunocytochemical analysis

Introduction

Diacylglycerol (DG) is a neutral lipid that is produced from various sources including phosphatidylinositol 4,5-bisphosphate, phosphatidylcholine, and triglyceride. Functionally, DG is not just an intermediate of lipid metabolism, but serves as a signaling molecule, which activates RasGRP, conventional and novel types of protein kinase C (PKC), Unc-13, and canonical transient receptor potential channels.[1,2] Within the cell, DG is distributed to the membranous structures, such as the plasma membrane, nuclear envelope, and endoplasmic reticulum membrane, and also to the nuclear matrix.[3] Therefore, DG metabolism should be tightly regulated to keep its levels within physiological range in the biological membranes and nucleus.[4]

One mechanism responsible for this regulation is an action of DG kinase (DGK), an enzyme that phosphorylates DG to produce phosphatidic acid (PA).[4,5] Because PA, a product of DGK, also serves as a second messenger activating hypoxia-inducible factor 1α, atypical PKCζ and mammalian target of Rapamycin, DGK is thought to control the switch between DG-mediated and PA-mediated pathways.

DGK constitutes an enzyme family composed of 10 isozymes in mammalian species.[2,5,6] Each isozyme has distinct properties of enzymatic activity, molecular structure, and subcellular localization.[6] Of the DGKs, DGKζ is classified into type IV DGK, which contains two EF-hand motifs (Ca^{2+} binding site), two Zn-fingers (DG-binding C1 domain), nuclear localization signal, and a catalytic domain.[7,8] It is reported that DGKζ is expressed ubiquitously in mammalian organs, including the brain, lung, liver, muscle, and pituitary gland.[9] Subcellular localization of DGKζ has been examined by cDNA transfection technique and immunohistochemistry on rat tissues by polyclonal antibody raised against rat DGKζ.[6,10] These studies show that DGKζ predominantly localizes to the nucleus, but in some occasions such as transient brain ischemia or kainite-induced seizure, DGKζ translocates from the nucleus to the cytoplasm in hippocampal neurons.[11,12] However, detailed morphological analysis of native DGKζ protein on human tissues or cells has not been reported yet, because no specific monoclonal antibody (mAb) is available to detect human DGKζ by immunostaining. In this study, we report a novel anti-human DGKζ mAb, DzMab-1, which is a very advantageous tool in immunocytochemical analysis of cultured human cells.

Materials and Methods

Cell lines

HeLa 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.,

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Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Antibiotics, including 100 U/mL of penicillin and 100 μg/mL of streptomycin were added to media.

Silencing RNA duplexes directed against human DGKζ (siDGKζ1, 5′-CCAGUCUUUCCUCUGGUAUCUCAAU-3′ and siDGKζ2 5′-GCCGCUUUCGGAAUAAGAUGUUCUA-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 human DGKζ or scrambled control using Lipofectamine RNAi MAX (Thermo Fisher Scientific, Inc.) according to manufacturer's instruction.

Plasmid preparation

Mouse DGKζ (mDGKζ) cDNA and human DGKζ (hDGKζ) cDNA were synthesized and subcloned into an expression vector, pMAL-c2 (New England Biolabs, Inc., Beverly, MA), with PA tag (GVAMPGAEDDVV)[13] using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan); the resultant construct was named pMAL-c2-mDGKζ-PA and pMAL-c2-hDGKζ-PA, respectively. The deletion mutants of hDGKζ produced using PCR were subcloned into pMAL-c2 with PA tag using the In-Fusion HD Cloning Kit. The substitution of hDGKζ amino acids 1–10 with alanine in dC200 of hDGKζ was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA). These constructs were verified using direct DNA sequencing.

Production of recombinant protein of DGKζ

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc.) were transformed with the plasmid pMAL-c2-DGKζ-PA. They were then cultured overnight at 37°C in the LB medium (Thermo Fisher Scientific, Inc.) containing 100 μg/mL ampicillin (Sigma-Aldrich Corp., St. Louis, MO). Cell pellets were resuspended in phosphate-buffered saline (PBS) with 1% Triton X-100 and 50 μg/mL aprotinin (Sigma-Aldrich Corp.). After sonication, the crude extracts were collected by centrifugation (9000 *g*, 30 minutes, 4°C). The lysates were passed through a 0.45 μm filter to remove any trace amounts of insoluble materials. Cleared lysates were mixed with NZ-1-Sepharose (1 mL bed volume) and incubated at 4°C for 2 hours under gentle agitation. The resin was then transferred to a column and washed with 20 mL Tris-buffered saline (pH 7.5). The bound protein was eluted with PA tag peptide at room temperature in a stepwise manner (1 mL × 10).

Hybridoma production

DzMab-1 was produced using rat medial iliac lymph node methods. The Animal Care and Use Committee of Tohoku University approved all animal experiments. In brief, WKY/Izm rats (Japan SLC, Inc., Shizuoka, Japan) were immunized by injecting 50 μg of pMAL-c2-mDGKζ-PA proteins together with Freund's complete adjuvant (Sigma-Aldrich Corp.) into the footpad. Additional immunization of 74 μg of pMAL-c2-mDGKζ-PA proteins were performed into the tailbase. The lymphocytes were fused with mouse myeloma Sp2/0-Ag14 cells using polyethylene glycol. The culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA) for the binding to pMAL-c2-mDGKζ-PA proteins. Then, second screening was performed using pMAL-c2-hDGKζ-PA.

Western blotting

Lysates were immunoprecipitated using a NZ-1-Sepharose and boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.). The samples were electrophoresed on 5%–20% polyacrylamide gels (Nacalai Tesque, Inc.) and transferred onto a polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) for 1 hour, the membrane was incubated with DzMab-1 or NZ-1 for 1 hour, followed by biotin-conjugated anti-rat immunoglobulin G (IgG) (1:1000 dilution; Agilent Technologies, Inc.) for 1 hour, and further incubated with VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes. The membrane was finally developed with the ImmunoStar LD Chemiluminescence Reagent (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) using the Sayaca-Imager (DRC Co., Ltd., Tokyo, Japan). All procedures of western blotting were performed at the room temperature.

Immunocytochemical analysis

HeLa cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes on ice. After fixation, cells were perforated with 0.3% Triton-X 100/PBS for 30 minutes at room temperature, followed by treatment with 5% bovine serum albumin (BSA) in PBS (BSA/PBS) to block nonspecific binding sites, and were incubated with 10 μg/mL of DzMab-1 or control (BSA/PBS) for overnight at room temperature in a moist chamber. They were incubated with goat anti-rat IgG-Alexa 488 (dilution 1:300; Thermo Fisher Scientific, Inc.) for 30 minutes at room temperature. Cells were also treated 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

Previously, immunohistochemical studies using rabbit polyclonal DGKζ antibody on rat tissues showed predominant localization of the immunoreactivity in the nuclei of various cell types, including neurons, hepatocytes, alveolar epithelial cells, and macrophages.[6] Nuclear localization of DGKζ immunoreactivity is consistent with cDNA tranfection studies in COS-7 cells and primary cultured neurons.[10,14]

As this antibody was not useful in immunohistochemical study on human tissues and cells, we aimed at obtaining a specific DGKζ antibody that is suitable for immunostaining of human cells. The recombinant maltose-binding protein (MBP)-mDGKζ-PA was captured using NZ-1-Sepharose and efficiently eluted with PA tag peptide. Rat was then immunized with MBP-mDGKζ-PA to develop novel anti-mDGKζ mAbs. Using ELISA, the culture supernatants of hybridomas were screened for the binding to MBP-mDGKζ-PA that was purified from *E. coli*. Furthermore, MBP-hDGKζ-PA was used as a second ELISA screening. As a result, DzMab-1 (rat

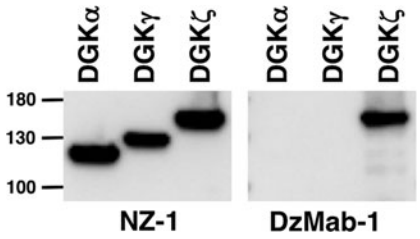

FIG. 1. Specificity of DzMab-1 for DGKζ. Immunoprecipitates of hDGKα, hDGKγ, and hDGKζ by anti-PA tag (NZ-1) were electrophoresed and transferred onto membranes. After blocking, the membranes were incubated with 10 μg/mL of DzMab-1 and 1 μg/mL of NZ-1, respectively. hDGK, human diacylglycerol kinase; PA, phosphatidic acid.

IgG_1, kappa) that can react with both MBP-mDGKζ-PA and MBP-hDGKζ-PA was established after limiting dilution. Western blot analysis demonstrated that DzMab-1 detected MBP-hDGKζ-PA but not MBP-hDGKα-PA or MBP-hDGKγ-PA (Fig. 1), indicating that DzMab-1 specifically reacts with hDGKζ.

In this study, we used HeLa cells, a human cervical cancer cell line, in which expression of DGKζ protein is reported.[15,16] Immunocytochemical analysis revealed that immunoreactivity for DzMab-1 is predominantly detected in the nucleus with a granular pattern of labeling and less intensely in the cytoplasm with a diffuse pattern (Fig. 2). Immunoreactivity observed in this study is similar to the one in previous studies using rabbit polyclonal antibody.[17,18]

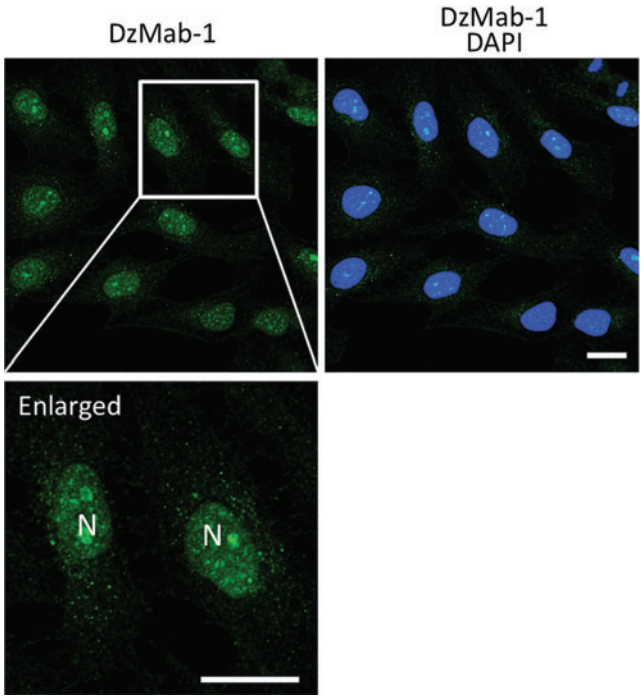

FIG. 2. Immunocytochemistry using DzMab-1 in HeLa cells. HeLa cells were stained with DzMab-1 antibody at a concentration of 10 μg/mL. Nuclear staining was performed with DAPI. Enlarged view (lower left panel) clearly shows granular staining in the nucleus (N) and diffuse staining in the cytoplasm. Bar = 10 μm.

Immunostaining without primary antibody abolished the immunoreactions (data not shown). Furthermore, we performed siRNA silencing experiment. As given in Figure 3, transfection with 20 pmol of siRNA for DGKζ (siDGKζ) significantly reduced the immunoreactivity for DzMab-1 compared with the transfection with scramble control siRNA (siControl). These results confirm that DzMab-1 reacts with native DGKζ protein specifically in human HeLa cells and that the images obtained with this antibody is authentic.

In addition, it should be mentioned that many of the nuclei in cells transfected with 40 pmol of siDGKζ are shrunk or irregular (Fig. 3, lower panels). Nuclear deformation is more evident in cells transfected with 60 pmol of siDGKζ (data not shown). No changes in the nuclear morphology were

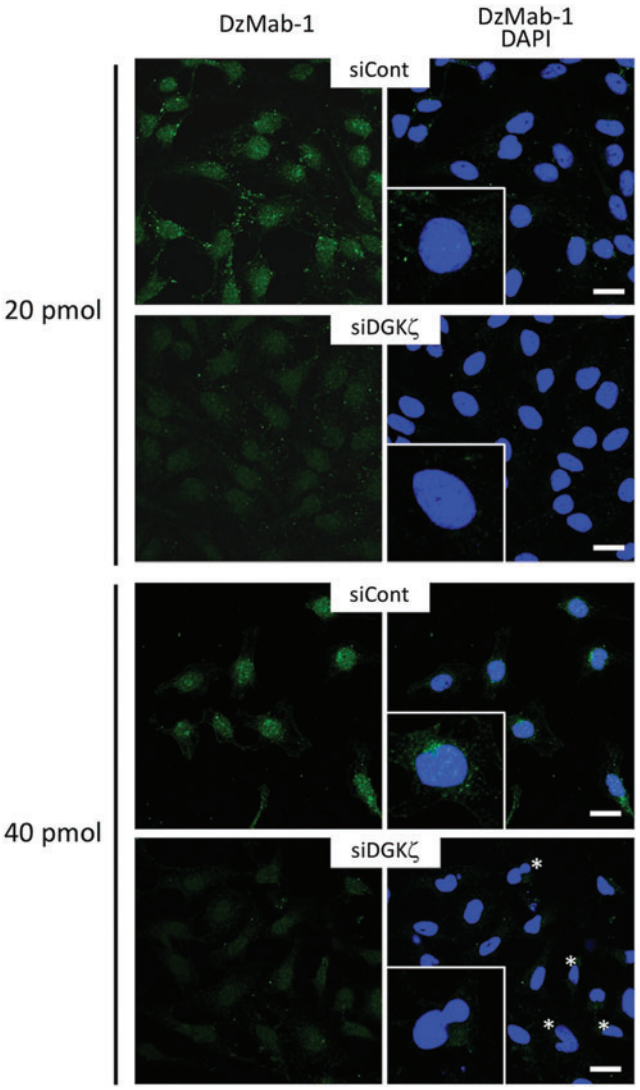

FIG. 3. Knockdown of DGKζ using siRNA in HeLa cells. Cells were transfected with siDGKζ at an amount of 20 pmol (upper panels) and 40 pmol/60 mm dish (lower panels). After 48 hours, immunocytochemistry was performed using DzMab-1. Note that shrunk or irregular nuclei are frequently observed in 40 pmol siDGKζ-treated cells (asterisks). AllStar Negative Control siRNA (siCont) was used as a control. Bar = 10 μm.

observed in cells transfected with siControl. These findings raise a possibility that reduced levels of DGKζ could induce nuclear deformability.

In this regard, we previously reported that DGKζ knockdown induces cleavage of PARP, an apoptotic marker, in HeLa cells after treatment with doxorubicin,[15] suggesting that DGKζ downregulation renders cells vulnerable to cell death in response to genotoxic stress. This seems to be correlated with the present findings that DGKζ knockdown induces nuclear deformation, showing that DGKζ is deeply involved in the regulation of nuclear morphology and function.

In conclusion, a novel anti-DGKζ mAb, DzMab-1, specifically reacts with native human DGKζ protein of HeLa cells in immunocytochemistry.

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Author Disclosure Statement

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