MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 37, Number 5, 2018

DOI: 10.1089/mab.2018.0026

Short Communications

DgMab-6: Antihuman DGKγ Monoclonal Antibody for Immunocytochemistry

Tomoyuki Nakano,[1] Satoshi Ogasawara,[2] Toshiaki Tanaka,[1] Yasukazu Hozumi,[3] Atsumi Yamaki,[2] Fumio Sakane,[2] Yasuhito Shirai,[4] Takuro Nakamura,[5] Miyuki Yanaka,[5] Shinji Yamada,[5] Mika K. Kaneko,[5] Yukinari Kato,[5,6] and Kaoru Goto[1]

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA). Since both DG and PA serve as lipidic second messengers, DGK plays a pivotal role in regulating the balance of two signaling pathways mediated by DG and PA in cellular functions. Reportedly, DGKγ, one of the 10 mammalian DGK isozymes, is involved in leukemic cell differentiation, mast cell function, and membrane traffic. Transfection studies using tagged expression vectors and immunohistochemistry on rat tissues revealed that DGKγ localizes to the cytoplasm, plasma membrane, and Golgi apparatus. However, a limited number of studies reported the detailed localization of native protein of DGKγ in human tissues and cells. In this study, we developed a novel anti-DGKγ monoclonal antibody, DgMab-6, which is very useful in immunocytochemistry of human cultured cells.

Keywords: DGKγ, monoclonal antibody, immunocytochemical analysis

Introduction

Diacylglycerol (DG) serves as a lipidic second messenger that activates various proteins containing DG-sensitive C1 domain, including conventional and novel types of protein kinase C (PKC), RasGRP, Unc-13, and canonical transient receptor potential channels.[1,2] Therefore, DG metabolism should be tightly regulated to keep its levels within physiological range. Of the DG metabolic pathways, diacylglycerol kinase (DGK) phosphorylates DG to produce phosphatidic acid (PA).[3,4] In addition to DG, PA is also revealed to serve as a messenger molecule activating hypoxia-inducible factor 1α, atypical PKCζ, and mammalian target of rapamycin. Thus, DGK is thought to control the switch between DG-mediated and PA-mediated pathways.

In mammalian species, DGK constitutes an enzyme family composed of 10 isozymes.[2,4,5] Each isozyme has a distinct molecular structure and subcellular localization pattern. Of DGKs, DGKγ cDNA was isolated in rat[6] and human[7] as the third DGK isozyme. DGKγ is classified into type I DGK, which contains two EF-hand motifs (Ca^{2+}-binding site), two Zn fingers (DG-binding C1 domain), and a catalytic domain. Reportedly, DGKγ is involved in a variety of cell functions including leukemic cell differentiation,[8] antigen-induced mast cell degranulation,[9] and membrane traffic between the endoplasmic reticulum and Golgi apparatus.[10] Analysis of DGKγ subcellular localization was performed mainly by cDNA transfection, which suggests that the subcellular localization of DGKγ is differentially regulated in cell species-dependent manner. In NIE-115 (a neuroblastoma cell line), DGKγ localizes to the cytoplasm and plasma membrane.[11] In U937 (a human promonocytic leukemic cell line), DGKγ localizes to the cytoplasm and translocates to the plasma membrane in response to TPA (12-*O*-tetradecanoyl phorbol-13 acetate), a membrane-permeant DG analogue typically used in cell-based studies.[8] In COS-7 (an African green monkey kidney fibroblast-like cell line), DGKγ is targeted to the Golgi apparatus, which is confirmed by double staining with a Golgi marker.[12]

Immunohistochemical examination using specific antibody against DGKγ is reported solely on rat tissues, including the brain, vascular endothelium, and adrenal cortex.[6,10,13] However, detailed morphological analysis of DGKγ native protein on human cells or tissues has not been reported yet, because no specific monoclonal antibody (mAb) is available

[1]Department of Anatomy and Cell Biology, Yamagata University Faculty of Medicine, Yamagata, Japan.
[2]Department of Chemistry, Graduate School of Science, Chiba University, Chiba, Japan.
[3]Department of Cell Biology and Morphology, Akita University Graduate School of Medicine, Akita, Japan.
[4]Department of Applied Chemistry in Bioscience, Graduate School of Agricultural Science, Kobe University, Kobe, Japan.
[5]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan.
[6]New Industry Creation Hatchery Center, Tohoku University, Sendai, Miyagi, Japan.

to detect human DGKγ by immunostaining. Herein, we report a novel antihuman DGKγ mAb, DgMab-6, which is very useful in immunocytochemical analysis of cultured cells.

Materials and Methods

Cell lines

P3U1 and HL-60 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in the RPMI 1640 medium (Nacalai Tesque, Inc., 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 units/mL of penicillin and 100 μg/mL of streptomycin were added to medium.

Silencing RNA duplexes directed against human DGKγ (siDGKγ1, 5′-CCACAUCUUACUGCCCACCUCCAUA-3′ and siDGKγ2 5′-CCACAGAGCUGAGGCCUAUAUU GAA-3′) were purchased from Thermo Fisher Scientific, Inc. Scrambled siRNA duplexes (AllStars Negative Control siRNA) (Qiagen, Valencia, CA) were used as control. HL-60 cells were transfected with siRNAs against human DGKγ or scrambled control using Lipofectamine RNAi MAX (Thermo Fisher Scientific Inc.) according to manufacturer's instruction.

Plasmid and recombinant protein of DGKγ

Human DGKγ cDNA was synthesized and was subcloned into an expression vector, pMAL-c2 (New England Biolabs, Inc., Beverly, MA), with PA tag (GVAMPGAEDDVV)[14] using the In-Fusion PCR cloning kit, named pMAL-c2-DGKγ-PA. This construct was verified by direct DNA sequencing.

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc.) were transformed with the plasmid pMAL-c2-DGKγ-PA. They were then cultured overnight at 37°C in the Luria broth 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 PBS. The bound protein was eluted with PA tag peptide at room temperature in a step-wise manner (1 mL × 10).

Hybridoma production

Female Balb/c mice (4 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Animals were housed under specific pathogen-free conditions. The ''Animal Care and Use Committee of Tohoku University'' approved the animal experiments described herein. Balb/c mice were immunized using an intraperitoneal (i.p.) injection of MBP-DGKγ-PA (100 μg) together with Imject Alum (Thermo Fisher Scientific, Inc.). After three additional immunizations, a booster injection was administered through i.p. 2 days before the spleen cells were harvested. The spleen cells were fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The fused cells were grown in the RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, Inc.).

Recombinant MBP-DGKγ-PA was immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 1 μg/mL for 30 minutes. After blocking with 1% bovine serum albumin in 0.05% Tween20/PBS, the plates were incubated with culture supernatant followed by 1:3000-diluted peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa Clara, CA). The enzymatic reaction was performed with a 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Immunocytochemical analysis

HL-60 cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) in a test tube for 20 minutes on ice. After fixation, cells were washed with PBS and were put on a slide glass as cell smear specimen. After brief air drying, cells were perforated with 0.3% Triton-X 100/PBS for 20 minutes at room temperature, followed by treatment with 5% normal goat serum in PBS (NGS/PBS) to block nonspecific binding sites, and were incubated with 5 μg/mL of DgMab-6 or control (NGS/PBS) for overnight at room temperature in a moist chamber. They were incubated with goat antimouse 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).

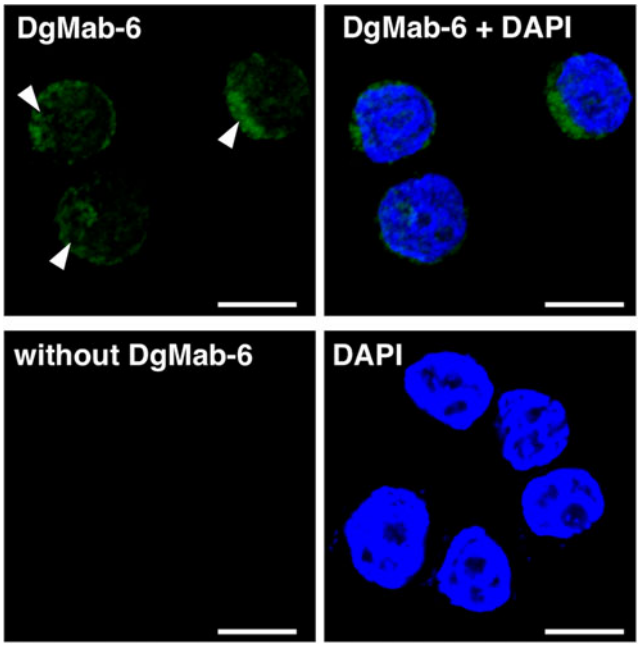

FIG. 1. Immunocytochemical analysis of human HL-60 cells using DgMab-6. Cells were stained with DgMab-6 at a concentration of 5 μg/mL. DAPI was used for nuclear staining. Arrowheads indicate immunoreactive site in the cytoplasm. As a negative control, cells were stained without primary antibody (lower panels). Scale bar = 10 μm.

Densitometry and statistical analysis

Fluorescent intensity was measured by ImageJ (National Institutes of Health, Bethesda, MD). Intensity per cell was expressed as mean ± standard error of the mean. Significance was determined by Welch's *t*-test at significance of $P < 0.001$.

Results and Discussion

In earlier studies, immunohistochemical examination using DGKγ antibody on rat tissues showed cytoplasmic localization of this isozyme in several neurons of the brain, including cerebellar Purkinje cells.[6,15] Subsequently, another polyclonal antibody against rat DGKγ was generated to examine subcellular localization of DGKγ on rat aortic endothelial cells.[10] By double immunostaining with organelle makers, immunoreactive site for this antibody was localized to the Golgi apparatus. A subsequent study revealed that the immunoreactivity is detected in the Golgi apparatus of rat adrenal cortex cells.[13] These ''Golgi patterns'' of localization are consistent with the data on transfected COS-7 cells.[12] However, morphological examination has not yet been performed on human cells or tissues since highly specific and sensitive mAbs against human DGKγ remained to be established.

In this study, the recombinant MBP-DGKγ-PA was captured using NZ-1-Sepharose and efficiently eluted with PA tag peptide.[14] Mice were then immunized with MBP-DGKγ-PA to develop novel anti-DGKγ mAbs. Using ELISA, the culture supernatants of hybridomas were screened for binding to recombinant MBP-DGKγ-PA that was purified from *E. coli*. As a result, DgMab-6 (mouse IgG_1, kappa) was established after limiting dilution. Immunoblot analysis demonstrated that DgMab-6 detected MBP-DGKγ-PA but not MBP-IDH1[16] (data not shown), indicating that DgMab-6 is specific against DGKγ in immunoblot analysis.

In this study, we used a human promonocytic leukemic cell line HL-60 cells in which mRNA expression of DGKγ was reported.[8] Immunocytochemical analysis revealed that immunoreactivity for DgMab-6 is detected as granular pattern in the cytoplasm (Fig. 1). This pattern of staining is similar to that in previous studies using rabbit polyclonal antibody,[10,13] suggesting Golgi pattern of distribution. At this moment, we failed to identify the immunoreactive structure as Golgi apparatus because of very narrow cytoplasmic space of HL-60 leukemic cells.

As a negative control, immunostaining without primary antibody abolished the immunoreactions, suggesting that the images obtained from DgMab-6 antibody are authentic. To further confirm the accuracy of the immunoreactions of DgMab-6, we performed siRNA silencing experiment. As shown in Figure 2, transfection with siRNA for DGKγ1 (siDGKγ1) significantly reduced the immunoreactivity compared with the transfection with scramble control siRNA (siControl). Similar results were obtained in the experiment using another siDGKγ2 (data not shown). These results confirm that DgMab-6 reacts with DGKγ specifically and correctly in human leukemic cell line HL-60 cells.

In conclusion, a novel anti-DGKγ mAb, DgMab-6, sensitively and specifically reacted with human DGKγ using immunocytochemical analysis. We could not observe specific bands of DGKγ using DgMab-6 in HL-60 cells by immunoblot analysis. We should further develop more useful anti-DGKγ mAbs for immunoblot or immunohistochemical analyses.

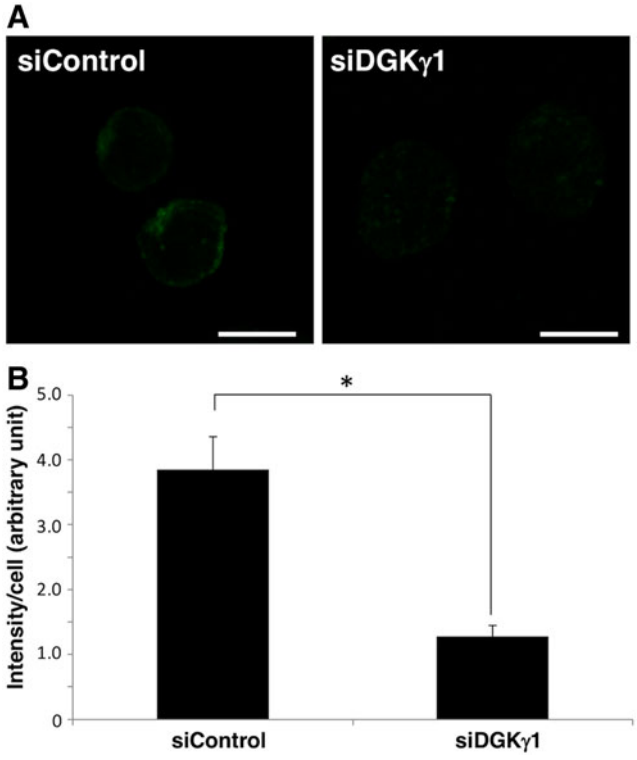

FIG. 2. Knockdown of DGKγ using siRNA. **(A)** Cells were transfected with human DGKγ siRNA (siDGKγ1). After 48 hours, immunocytochemistry was performed with DgMab-6. AllStars Negative Control siRNA (siControl) was used as a control. Scale bar = 10 μm. **(B)** Densitometry of the immunoreactivity on a single cell was carried out by NIH-image J. Data (intensity/cell) are represented as mean ± SE ($n = 30$). *$P < 0.001$. DGK, diacylglycerol kinase.

Acknowledgments

This work was supported, in part, by AMED under Grant Number JP18am0101078 (Y.K.) and by the Grant-in-Aid for Challenging Exploratory Research from MEXT (T.N. and K.G.).

Author Disclosure Statement

K.G., Y.S., F.S., and Y.K. received research funding from Ono Pharmaceutical Co., Ltd. The other authors have no conflict of interest.

References

1. Kanoh H, Yamada K, and Sakane F: Diacylglycerol kinase: A key modulator of signal transduction? Trends Biochem Sci 1990;15:47–50.
2. Topham MK, and Epand RM: Mammalian diacylglycerol kinases: Molecular interactions and biological functions of selected isoforms. Biochim Biophys Acta 2009;1790:416–424.
3. Goto K, Hozumi Y, and Kondo H: Diacylglycerol, phosphatidic acid, and the converting enzyme, diacylglycerol

kinase, in the nucleus. Biochim Biophys Acta 2006;1761: 535–541.
4. Sakane F, Imai S, Kai M, Yasuda S, and Kanoh H: Diacylglycerol kinases: Why so many of them? Biochim Biophys Acta 2007;1771:793–806.
5. Goto K, Hozumi Y, Nakano T, Saino SS, and Kondo H: Cell biology and pathophysiology of the diacylglycerol kinase family: Morphological aspects in tissues and organs. Int Rev Cytol 2007;264:25–63.
6. Goto K, Funayama M, and Kondo H: Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum. Proc Natl Acad Sci U S A 1994;91:13042–13046.
7. Kai M, Sakane F, Imai S, Wada I, and Kanoh H: Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in human retina with a truncated and inactive enzyme expression in most other human cells. J Biol Chem 1994;269:18492–18498.
8. Yamada K, Sakane F, Imai S, Tsushima S, Murakami T, and Kanoh H: Regulatory role of diacylglycerol kinase gamma in macrophage differentiation of leukemia cells. Biochem Biophys Res Commun 2003;305:101–107.
9. Sakuma M, Shirai Y, Ueyama T, and Saito N: Diacylglycerol kinase gamma regulates antigen-induced mast cell degranulation by mediating Ca^{2+} influxes. Biochem Biophys Res Commun 2014;445:340–345.
10. Nakano T, Hozumi Y, Goto K, and Wakabayashi I: Involvement of diacylglycerol kinase gamma in modulation of iNOS synthesis in Golgi apparatus of vascular endothelial cells. Naunyn Schmiedebergs Arch Pharmacol 2012; 385:787–795.
11. Tanino F, Maeda Y, Sakai H, and Sakane F: Induction of filopodia-like protrusions in N1E-115 neuroblastoma cells by diacylglycerol kinase gamma independent of its enzymatic activity: Potential novel function of the C-terminal region containing the catalytic domain of diacylglycerol kinase gamma. Mol Cell Biochem 2013;373:85–93.
12. Kobayashi N, Hozumi Y, Ito T, Hosoya T, Kondo H, and Goto K: Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. Eur J Cell Biol 2007;86:433–444.
13. Hozumi Y, Akimoto R, Suzuki A, Otani K, Watanabe M, and Goto K: Expression and localization of the diacylglycerol kinase family and of phosphoinositide signaling molecules in adrenal gland. Cell Tissue Res 2015;362:295–305.
14. Fujii Y, Kaneko M, Neyazaki M, Nogi T, Kato Y, and Takagi J: PA tag: A versatile protein tagging system using a super high affinity antibody against a dodecapeptide derived from human podoplanin. Protein Expr Purif 2014;95:240–247.
15. Adachi N, Oyasu M, Taniguchi T, Yamaguchi Y, Takenaka R, Shirai Y, and Saito N: Immunocytochemical localization of a neuron-specific diacylglycerol kinase beta and gamma in the developing rat brain. Brain Res Mol Brain Res 2005; 139:288–299.
16. Nakano T, Ogasawara S, Tanaka T, Hozumi Y, Mizuno S, Satoh E, Sakane F, Okada N, Taketomi A, Honma R, Nakamura T, Saidoh N, Yanaka M, Itai S, Handa S, Chang YW, Yamada S, Kaneko MK, Kato Y, and Goto K: DaMab-2: Anti-human DGKalpha monoclonal antibody for immunocytochemistry. Monoclon Antib Immunodiagn Immunother 2017;36:181–184.

Address correspondence to:
Kaoru Goto
Department of Anatomy and Cell Biology
Yamagata University Faculty of Medicine
2-2-2 Iida-nishi
Yamagata 990-9585
Japan

E-mail: kgoto@med.id.yamagata-u.ac.jp

Received: June 28, 2018
Accepted: September 22, 2018