

# DaMab-2: Anti-Human DGK $\alpha$ Monoclonal Antibody for Immunocytochemistry

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Diacylglycerol kinase (DGK) is responsible for the enzymatic conversion of diacylglycerol to phosphatidic acid. Since both diacylglycerol and phosphatidic acid serve as signaling molecules, DGK is regarded as a hub between diacylglycerol-mediated and phosphatidic acid-mediated signaling. One of the 10 DGK isozymes, DGK $\alpha$ , is shown to be involved in T cell function. Transfection studies using tagged expression vectors revealed that DGK $\alpha$  localizes to the cytoplasm and nucleus and translocates to the plasma membrane in response to T cell receptor stimulation. However, a limited number of studies reported the localization of native protein of DGK $\alpha$  in tissues and cells. In this study, we immunized mice with recombinant DGK $\alpha$  and developed several anti-DGK $\alpha$  monoclonal antibodies (mAbs). One of the established anti-DGK $\alpha$  mAbs is a clone DaMab-2 (mouse IgG<sub>1</sub>, kappa). In enzyme-linked immunosorbent assay, DaMab-2 recognized only DGK $\alpha$ , and did not react with the other isozymes, such as DGK $\gamma$ , DGK $\zeta$ , DGK $\eta$ , and DGK $\delta$ . Importantly, DaMab-2 is very useful in immunocytochemical analysis of human cultured cells, indicating that DaMab-2 is advantageous to analyze the localization and function of DGK $\alpha$ .

**Keywords:** DGK $\alpha$ , monoclonal antibody, immunocytochemical analysis

## Introduction

**D**IACYLGLYCEROL KINASE (DGK) is an enzyme that phosphorylates diacylglycerol to produce phosphatidic acid.<sup>(1,2)</sup> Diacylglycerol is a neutral lipid derived from various sources, such as phosphatidylinositol 4, 5-bisphosphate and phosphatidylcholine. Diacylglycerol serves as a second messenger, which activates conventional and novel types of the protein kinase C (PKC) family, RasGRP, Unc-13, and canonical transient receptor potential channels.<sup>(1,3)</sup> In addition, phosphatidic acid is also shown to act as a messenger molecule activating hypoxia-inducible factor 1 $\alpha$ , atypical PKC $\zeta$ , and mammalian target of Rapamycin. Thus, DGK is thought to serve as a signaling hub between diacylglycerol-mediated and phosphatidic acid-mediated pathways.

DGK constitutes an enzyme family composed of 10 isozymes in mammalian species.<sup>(1-3)</sup> Each isozyme has a distinct molecular structure and subcellular localization pattern.

Of DGKs, 80-kDa DGK $\alpha$  is the first identified enzyme, which contains EF-hand motif (a Ca<sup>2+</sup>-binding site), Zn-finger (C1 domain, diacylglycerol-binding site), and catalytic domain. A wide range of cell types and tissues express DGK $\alpha$ , including lymphocytes, glial cells, vascular cells, hepatocytes, adrenal medullary cells, fibroblasts, sarcoma cells, and melanoma cells.<sup>(3-11)</sup>

Functional significance of DGK $\alpha$  has been intensely investigated in T lymphocytes. In T cells, DGK $\alpha$  regulates cell proliferation in response to IL-2 stimulation<sup>(3)</sup> and is involved in T cell receptor (TCR) signaling through modulating RasGRP activity.<sup>(12)</sup> T cells isolated from DGK $\alpha$ -deficient mice show altered activity of TCR signaling and hyperproliferation.<sup>(13)</sup> Transfection studies in T cells demonstrated that DGK $\alpha$  localizes in the cytoplasm and translocates to the plasma membrane in response to the external stimuli.<sup>(14)</sup>

A limited number of studies have been reported about the localization of native protein using specific monoclonal

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antibodies (mAbs) because no mAb is available to detect human DGK $\alpha$  by immunostaining. Herein, we report a novel anti-human DGK $\alpha$  mAb, DaMab-2, which is very useful in immunocytochemical analysis of cultured cells.

## Materials and Methods

### Cell lines

P3 U1 and HeLa were obtained from the American Type Culture Collection (Manassas, VA). P3 U1 was cultured in the RPMI 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. HeLa was cultured in DMEM (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% FBS. Antibiotics, including 100 U/mL of penicillin and 100  $\mu$ g/mL of streptomycin were added to media.

### Plasmid preparation

Human DGK $\alpha$  cDNA<sup>(15)</sup> was synthesized, and subcloned into an expression vector, pMAL-c2 (New England Biolabs, Inc., Beverly, MA), with PA tag (GVAMPGAEDDVV)<sup>(16–18)</sup> using the In-Fusion PCR Cloning Kit, named pMAL-c2-DGK $\alpha$ -PA. DGK $\alpha$  with PA tag was also subcloned into an expression vector, pET41a(+) (Novagen; EMD Millipore Corp., Billerica, MA), with PA tag of N-terminus and RAP tag (DMVNPGLDRIE)<sup>(19)</sup> of C-terminus, named pET41a-PA-DGK $\alpha$ -RAP. This construct was verified by direct DNA sequencing.

### Production of recombinant protein of DGK $\alpha$

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc.) were transformed with the plasmid pMAL-c2-DGK $\alpha$ -PA. They were then cultured overnight at 37°C in the LB medium (Thermo Fisher Scientific, Inc.) containing 100  $\mu$ g/mL ampicillin (Sigma-Aldrich Corp., St. Louis, MO). Cell pellets were resuspended in phosphate-buffered saline (PBS) with 1% Triton X-100 and 50  $\mu$ g/mL aprotinin (Sigma-Aldrich Corp.). After sonication, the crude extracts were collected by centrifugation (9000 g, 30 min, 4°C). The recombinant PA-DGK $\alpha$ -RAP was expressed in transformed *E. coli* BL21 (DE3) cells that were cultured for 3 h at 37°C in the presence of 1 mM of IPTG, and the bacterial pellet from a 400 mL culture was suspended in 10 mL TBS (pH 7.5). Bacteria were lysed by sonication, and the soluble fraction was obtained by centrifugation at 15,000 g for 15 min.

The lysates were passed through a 0.45- $\mu$ m 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 h under gentle agitation. The resin was then transferred to a column and washed with 20 mL TBS (pH 7.5). The bound protein was eluted with PA tag peptide at room temperature in a stepwise manner (1 mL  $\times$  10).

### Hybridoma production

Female BALB/c mice (4 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Animals were housed under 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-DGK $\alpha$ -PA (100  $\mu$ g) together with Imject Alum (Thermo Fisher Scientific, Inc.). After three additional immunizations, a booster injection was administered through i.p. two days before the spleen cells were harvested. The spleen cells were fused with P3 U1 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.). Culture supernatants were screened using enzyme-linked immunosorbent assay (ELISA) for binding to recombinant MBP-DGK $\alpha$ -PA and PA-DGK $\alpha$ -RAP.

### Enzyme-linked immunosorbent assay

Recombinant MBP-DGK $\alpha$ -PA and PA-DGK $\alpha$ -RAP were immobilized on Nunc MaxiSorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at a concentration of 1  $\mu$ g/mL for 30 min. 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 (Dako, Glostrup, Denmark). The enzymatic reaction was performed with a 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). The optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA).

### Transfection of siRNA against human DGK $\alpha$

Silencing RNA duplexes directed against human DGK $\alpha$  (siDGK $\alpha$ 1, 5'-UUAGGAGGUUGAACACCUGUCGAGG-3' and siDGK $\alpha$ 2 5'-CCUGGCCUCUGGACCGGAUCG UAAA-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 DGK $\alpha$  or scrambled control using Lipofectamine RNAi MAX (Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. After 48 h of transfection, cells were fixed and stained by DaMab-2 as described above.

### Immunocytochemical analysis

HeLa cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min on ice. Cells were perforated with 0.3% Triton-X 100/PBS for 20 min at room temperature, followed by treatment with 5% normal goat serum in PBS (NGS/PBS) to block nonspecific binding sites, and were incubated with 20  $\mu$ g/mL of DaMab-2 or control (NGS/PBS) overnight at room temperature in a moist chamber. They were incubated with goat anti-mouse IgG-Alexa 488 (dilution 1:300; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. Cells were also treated with DAPI (Thermo Fisher Scientific, Inc.) to stain cell nuclei. They were examined using confocal laser scanning microscopy (LSM700; Carl Zeiss, Inc., Jena, Germany).

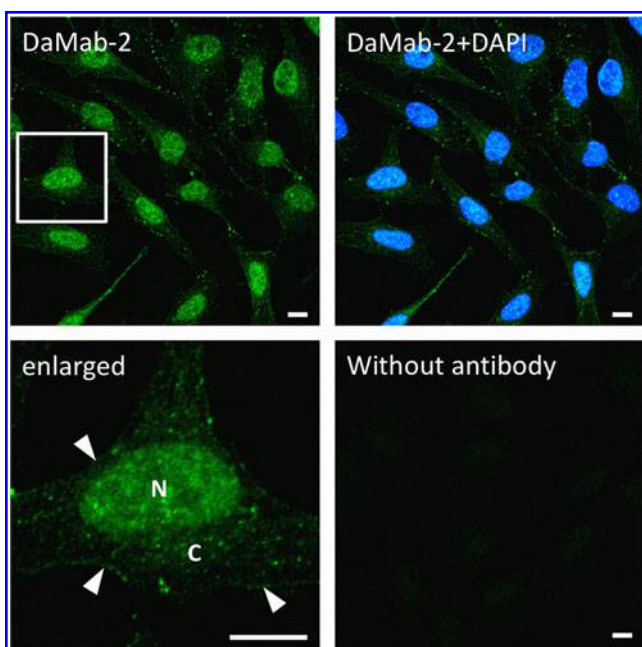
## Results

The recombinant MBP-DGK $\alpha$ -PA was captured using NZ-1-Sepharose and efficiently eluted with PA tag peptide.<sup>(16–18)</sup> Mice were then immunized with MBP-DGK $\alpha$ -PA to develop novel anti-DGK $\alpha$  mAbs. Using ELISA, the culture supernatants of hybridomas were screened for binding to recombinant MBP-

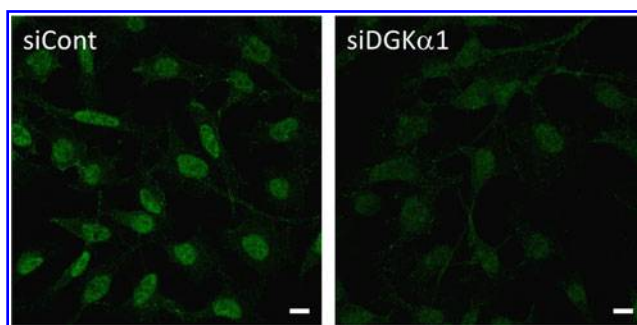
DGK $\alpha$ -PA or PA-DGK $\alpha$ -RAP that was purified from *E. coli*. As a result, DaMab-2 (mouse IgG<sub>1</sub>, kappa) was established after limiting dilution. Western blot analysis demonstrated that DaMab-2 detected MBP-DGK $\alpha$ -PA, but not MBP-IDH1,<sup>(20–22)</sup> indicating that DaMab-2 reacts with DGK $\alpha$ , but does not react with MBP tag in western blot analysis (Supplementary Fig. S1). ELISA demonstrated that DaMab-2 reacted only with DGK $\alpha$ , and did not react with the other isozymes, such as DGK $\gamma$ , DGK $\zeta$ , DGK $\eta$ , and DGK $\delta$  (data not shown).

Immunocytochemical analyses revealed that DaMab-2 reacted with HeLa cells (Fig. 1). The enlarged view clearly demonstrated a diffuse staining pattern in both the nucleus and cytoplasm. This pattern of staining was identical to the previous studies using rabbit polyclonal antibody on rat aortic smooth muscle and endothelial cells.<sup>(8,9)</sup> In addition, DaMab-2 reveals faint immunoreactions in the plasma membrane. Previous cDNA transfection studies described that DGK $\alpha$  translocates to the plasma membrane in response to TCR stimulation.<sup>(14)</sup> Negative control (without primary antibody) showed an absence of immunoreactions, suggesting that images obtained from DaMab-2 antibody were authentic.

To further confirm the accuracy of the immunoreactions of DaMab-2, we performed siRNA silencing experiments. As shown in Figure 2, transfection with siDGK $\alpha$ 1 significantly reduced the immunoreactivity compared with the transfected scramble control siRNA (siCont). Similar results were obtained in the experiment using siDGK $\alpha$ 2 (data not shown). These results confirm that DaMab-2 reacts with DGK $\alpha$  specifically and correctly in human cell line HeLa cells.



**FIG. 1.** Immunocytochemical analysis using DaMab-2. HeLa cells were stained with DaMab-2 at a concentration of 20  $\mu$ g/mL. Nuclear staining was performed with DAPI. As a negative control, cells were stained without primary antibody (lower right). Enlarged view of the square is shown on the lower left panel. N; Nucleus, C; Cytoplasm, arrowheads; plasma membrane. Bar = 10  $\mu$ m.



**FIG. 2.** Knockdown of DGK $\alpha$  using siRNA. DaMab-2 immunocytochemistry was performed after siRNA treatment for human DGK $\alpha$  (siDGK $\alpha$ 1). AllStars Negative Control siRNA (siCont) was used as a control. Bar = 10  $\mu$ m.

## Discussion

DGK $\alpha$  protein was purified from porcine brain in 1983,<sup>(23)</sup> followed by cDNA cloning from porcine,<sup>(24)</sup> human,<sup>(15)</sup> and rat brains.<sup>(4)</sup> In the rat study, a rabbit polyclonal antibody against rat DGK $\alpha$  was generated to determine DGK $\alpha$ -expressing cells in the rat brain. Fibrous and dot-like immunoreactions were detected in white matter by the polyclonal antibody, suggestive of an oligodendrocyte pattern. Using this polyclonal antibody, DGK $\alpha$  was shown to localize diffusely in the cytoplasm of hepatocytes and medullary chromaffin cells,<sup>(10,11)</sup> and in both the nucleus and cytoplasm of vascular cells<sup>(8,9)</sup> of rat tissues. However, highly specific and sensitive mAbs against human DGK $\alpha$  remain to be established.

Herein, we immunized mice with recombinant DGK $\alpha$  and successfully developed a clone DaMab-2 (mouse IgG<sub>1</sub>, kappa). ELISA demonstrated that DaMab-2 recognized only DGK $\alpha$ , and did not react with the other isozymes, such as DGK $\gamma$ , DGK $\zeta$ , DGK $\eta$ , and DGK $\delta$  (data not shown). Furthermore, DaMab-2 is very useful in immunocytochemical analysis of HeLa cells (Fig. 1). Unfortunately, DaMab-2 did not react with endogenous DGK $\alpha$  of HeLa cells in western blot (data not shown). In this study, we focused on the development of mAbs against DGK $\alpha$  for immunocytochemistry. It might often be difficult to establish mAbs, which are useful for both western blot analysis and immunocytochemical analysis. The design of immunogen and screening methods to produce specific mAbs are totally different when we aim to develop mAbs for western blot or immunocytochemical analysis. In particular, it is very difficult to produce specific and sensitive mAbs to detect endogenous proteins in every experiment. Indeed, DaMab-2 can detect recombinant DaMab-2 in western blot (Supplementary Fig. S1), although it did not react with endogenous DGK $\alpha$  of HeLa cells in western blot. Several anti-DGK $\alpha$  mAbs are commercially available, and have been reported to be useful in western blot analysis and immunocytochemical analysis. However, recombinant or overexpressed DGK $\alpha$  are usually used in those studies. We need to further develop clones for detecting endogenous DGK $\alpha$  in western blot analysis.

In conclusion, a novel anti-DGK $\alpha$  mAb, DaMab-2, sensitively and specifically reacted with human DGK $\alpha$  using immunocytochemical analyses. DaMab-2 could be advantageous to analyze the localization and function of DGK $\alpha$ .

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## Author Disclosure Statement

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