

# Epitope Mapping of Antidiacylglycerol Kinase $\alpha$ Monoclonal Antibody DaMab-2

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Diacylglycerol kinase (DGK) is responsible for the enzymatic conversion of diacylglycerol (DG) to phosphatidic acid (PA). Both DG and PA serve as signaling molecules; therefore, DGK functions as a key enzyme between DG- and PA-mediated signaling. DGK $\alpha$ , one of the 10 DGK isozymes, is involved in T cell function and has been shown to localize in the cytoplasm and nucleus. Furthermore, DGK $\alpha$  translocates to the plasma membrane in response to T cell receptor stimulation. Recently, we developed a specific monoclonal antibody (mAb), DaMab-2 (mouse IgG<sub>1</sub>, kappa), against DGK $\alpha$ . DaMab-2 is very useful in immunocytochemical analysis using HeLa cells. In this study, we characterized the binding epitope of DaMab-2 using Western blot and revealed that Cys246, Lys249, Pro252, and Cys253 of DGK $\alpha$  are important for DaMab-2 binding to the DGK $\alpha$  protein. Our findings can be applied for the production of more functional anti-DGK $\alpha$  mAbs.

**Keywords:** diacylglycerol kinase, DGK $\alpha$ , monoclonal antibody, epitope mapping

## Introduction

**D**IACYLGLYCEROL KINASE (DGK) phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA).<sup>(1,2)</sup> DG is a neutral lipid derived from various sources, including phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine, and serves as a second messenger that activates the conventional and novel types of the protein kinase C (PKC) family, RasGRP, Unc-13, and canonical transient receptor potential channels.<sup>(2,3)</sup> PA acts as a messenger molecule that activates hypoxia-inducible factor-1 $\alpha$ , atypical PKC $\zeta$ , and mammalian target of rapamycin. Therefore, DGK serves as a key enzyme between DG- and PA-mediated pathways.

In mammalian species, DGK constitutes an enzyme family comprising 10 isozymes.<sup>(1,2)</sup> Each isozyme possesses a distinct molecular structure and subcellular localization pattern. DGK $\alpha$  is the first identified enzyme of 80 kDa that contains an EF-hand motif (Ca<sup>2+</sup>-binding site), a Zn finger (C1 domain, DG-binding site), and a catalytic domain. Various cell types and tissues express DGK $\alpha$  in normal cells, including glial cells, vascular cells, hepatocytes, adrenal medullary cells, fibroblasts, and lymphocytes, or in several cancer cells, such as melanomas and sarcomas.<sup>(3–11)</sup> DGK $\alpha$  regulates cell proliferation in response to IL-2 stimulation in T cells<sup>(3)</sup> and is involved in T cell receptor (TCR) signaling by modulating RasGRP activity.<sup>(12)</sup> T cells isolated from DGK $\alpha$ -deficient

mice show an altered activity of TCR signaling and hyperproliferation.<sup>(13)</sup> DGK $\alpha$  localizes in the cytoplasm and translocates to the plasma membrane in response to external stimuli.<sup>(14)</sup>

Because no specific anti-DGK $\alpha$  monoclonal antibody (mAb) was available to detect human DGK $\alpha$  by immunostaining, the localization of the native protein was not clarified. Recently, we reported a novel antihuman DGK $\alpha$  mAb, DaMab-2 (mouse IgG<sub>1</sub>, kappa), which is very useful in immunocytochemical analysis using HeLa cells. In this study, we characterized the binding epitope of DaMab-2 using Western blot.

## Materials and Methods

### 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)</sup> using the In-Fusion HD Cloning kit (Takara Bio, Inc., Shiga, Japan), named pMAL-c2-DGK $\alpha$ -PA. The deletion mutants of DGK $\alpha$  were produced by polymerase chain reaction (PCR) and subcloned into pMAL-c2 with PA tag using the In-Fusion HD Cloning kit. The substitution of DGK $\alpha$  amino acids 240–260 with either alanine or glycine in dC320 of DGK $\alpha$  was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa

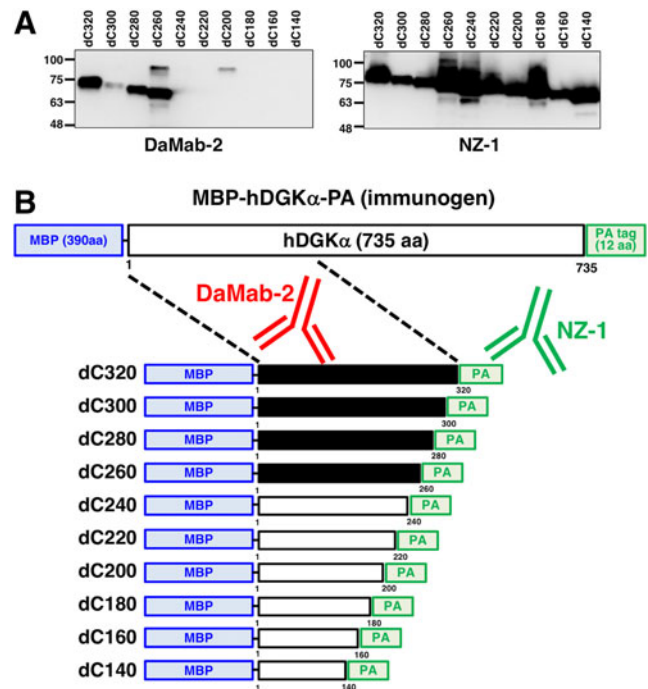
Clara, CA). These constructs were verified by direct DNA sequencing.

### Western blot analyses

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc., Waltham, MA) were transformed and cultured overnight at 37°C in Luria-Bertani (LB) medium (Thermo Fisher Scientific) containing 100 µg/mL ampicillin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Cell pellets were resuspended in phosphate-buffered saline with 1% Triton X-100 and 50 µg/mL aprotinin (Sigma-Aldrich Corp.). Lysates were immunoprecipitated using amylose resin (New England Biolabs, Inc.) and boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc., Kyoto, Japan). The samples were electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto a polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) for 1 h at room temperature, the membrane was incubated with DaMab-2<sup>(17)</sup> overnight at 4°C, NZ-1 (anti-PA tag) for 1 h at room temperature, or LpMab-13 (anti-PA tag) for 1 h at room temperature<sup>(18)</sup> and then with peroxidase-conjugated anti-mouse IgG (1:2000 dilution; Agilent Technologies, Inc.) or antirat IgG (1:10,000 dilution; Sigma-Aldrich Corp.) for 1 h at room temperature. The membrane was finally developed using ImmunoStar LD Chemiluminescence Reagent (FUJIFILM Wako Pure Chemical Corporation) using Sayaca-Imager (DRC Co., Ltd., Tokyo, Japan).

### Results and Discussion

Several anti-DGKα mAbs are commercially available and have been reported to be useful in Western blot and immunocytochemical analyses. However, recombinant or over-expressed DGKα has been usually used in these studies.

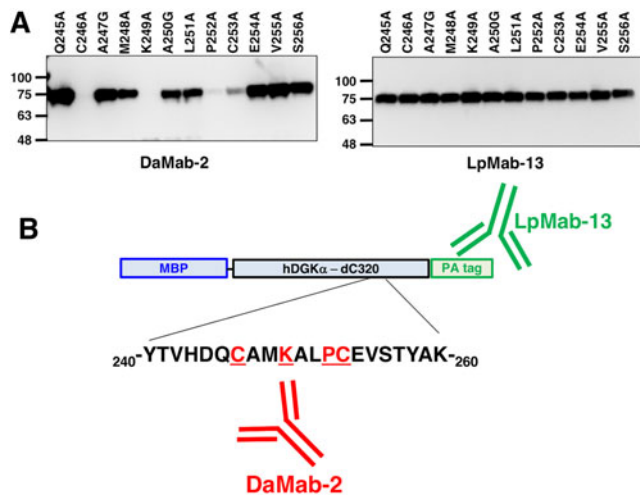


**FIG. 1.** Production of DGKα deletion mutants. **(A)** Immunoprecipitates of deletion mutants were electrophoresed and transferred onto PVDF membranes. After blocking, membranes were incubated with 20 µg/mL DaMab-2 or 1 µg/mL anti-PA tag (NZ-1) followed by peroxidase-conjugated antimouse or antirat IgG. **(B)** Schematic illustration of DaMab-2 epitope. Black bars, deletion mutants detected by DaMab-2; white bars, deletion mutants not detected by DaMab-2. hDGKα, human diacylglycerol kinase α; MBP, maltose-binding protein; PA, phosphatidic acid; PVDF, polyvinylidene difluoride.

TABLE 1. EPIOTOPE MAPPING OF DAMAB-2 USING POINT MUTANTS

Point mutants	Sequence	DaMab-2	LpMab-13
Y240A	ATVHDQCAMKALPCEVSTYAK	+	+
T241A	YAVHDQCAMKALPCEVSTYAK	+	+
V242A	YTAHDQCAMKALPCEVSTYAK	+	+
H243A	YTVADQCAMKALPCEVSTYAK	+	+
D244A	YTVHAQCAMKALPCEVSTYAK	+	+
Q245A	YTVHDACAMKALPCEVSTYAK	+	+
C246A	YTVHDQAAMKALPCEVSTYAK	–	+
A247G	YTVHDQCGMKALPCEVSTYAK	+	+
M248A	YTVHDQCAAKALPCEVSTYAK	+	+
K249A	YTVHDQCAMAALPCEVSTYAK	–	+
A250G	YTVHDQCAMKGLPCEVSTYAK	+	+
L251A	YTVHDQCAMKAAPCEVSTYAK	+	+
P252A	YTVHDQCAMKALACEVSTYAK	–	+
C253A	YTVHDQCAMKALPAEVSTYAK	–	+
E254A	YTVHDQCAMKALPCAVSTYAK	+	+
V255A	YTVHDQCAMKALPCEASTYAK	+	+
S256A	YTVHDQCAMKALPCEVATYAK	+	+
T257A	YTVHDQCAMKALPCEVSAYAK	+	+
Y258A	YTVHDQCAMKALPCEVSTAAYAK	+	+
A259G	YTVHDQCAMKALPCEVSTYGYAK	+	+
K260A	YTVHDQCAMKALPCEVSTYAA	+	+

Italic letters show the substitution of amino acid.



**FIG. 2.** Epitope mapping of DaMab-2 using deletion and point mutants of DGK $\alpha$ . **(A)** Immunoprecipitates of point mutants were electrophoresed and transferred onto PVDF membranes. After blocking, membranes were incubated with 20  $\mu$ g/mL DaMab-2 or 1  $\mu$ g/mL anti-PA tag (LpMab-13) followed by peroxidase-conjugated antimouse IgG. **(B)** Schematic illustration of DaMab-2 epitope. Red underlined letters, the critical amino acids for DaMab-2 recognition.

Recently, we immunized mice with recombinant DGK $\alpha$  and developed a clone (DaMab-2).<sup>(17)</sup> DaMab-2 recognized only DGK $\alpha$  in enzyme-linked immunosorbent assay and did not react with other isozymes, such as DGK $\gamma$ , DGK $\zeta$ , DGK $\eta$ , and DGK $\delta$ . Furthermore, DaMab-2 is very useful in immunocytochemical analysis using HeLa cells.

As shown in Figure 1, we produced 10 C-terminal deletion mutants of DGK $\alpha$  (dC320, dC300, dC280, dC260, dC240, dC220, dC200, dC180, dC160, and dC140). Western blot demonstrated that DaMab-2 detected dC320, dC300, dC280, and dC260 but not dC240, dC220, dC200, dC180, dC160, and dC140, although all deletion mutants were detected by an anti-PA tag mAb, NZ-1 (Fig. 1A), indicating that the C-terminus of the DaMab-2-epitope exists between amino acids 240 and 260. These results are summarized in Figure 1B.

We next produced the following 21 DGK $\alpha$  point mutants: Y240A, T241A, V242A, H243A, D244A, Q245A, C246A, A247G, M248A, K249A, A250G, L251A, P252A, C253A, E254A, V255A, S256A, T257A, Y258A, A259G, and K260A (Table 1). Western blot demonstrated that the anti-PA tag mAb, LpMab-13, detected Q245A, C246A, A247G, M248A, K249A, A250G, L251A, P252A, C253A, E254A, V255A, and S256A (Fig. 2A). In contrast, DaMab-2 strongly detected Q245A, A247G, M248A, A250G, L251A, E254A, V255A, and S256A, but did not detect mutants C246A and K249A and weakly detected P252A and C253A (Fig. 2A). Both DaMab-2 and LpMab-13 recognized the other point mutants, such as Y240A, T241A, V242A, H243A, D244A, T257A, Y258A, A259G, and K260A (Table 1). DaMab-2 epitope is summarized in Figure 2B.

In conclusion, Cys246, Lys249, Pro252, and Cys253 are important for DaMab-2 binding to the DGK $\alpha$  protein. Our findings can be applied for the production of more functional anti-DGK $\alpha$  mAbs.

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### Author Disclosure Statement

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