Epitope Mapping of Antidiacylglycerol Kinase α Monoclonal Antibody DaMab-2

Masato Sano, Mika K. Kaneko, and Yukinari Kato

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 α , 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 α translocates to the plasma membrane in response to T cell receptor stimulation. Recently, we developed a specific monoclonal antibody (mAb), DaMab-2 (mouse IgG₁, kappa), against DGK α . 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 α are important for DaMab-2 binding to the DGK α protein. Our findings can be applied for the production of more functional anti-DGK α mAbs.

Keywords: diacylglycerol kinase, DGK α , monoclonal antibody, epitope mapping

Introduction

DIACYLGLYCEROL KINASE (DGK) phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA).^(1,2) 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.^(2,3) PA acts as a messenger molecule that activates hypoxia-inducible factor-1 α , atypical PKC ζ , 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.^(1,2) Each isozyme possesses a distinct molecular structure and subcellular localization pattern. DGK α is the first identified enzyme of 80 kDa that contains an EF-hand motif (Ca²⁺-binding site), a Zn finger (C1 domain, DG-binding site), and a catalytic domain. Various cell types and tissues express DGK α 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.^(3–11) DGK α regulates cell proliferation in response to IL-2 stimulation in T cells⁽³⁾ and is involved in T cell receptor (TCR) signaling by modulating RasGRP activity.⁽¹²⁾ T cells isolated from DGK α -deficient

mice show an altered activity of TCR signaling and hyperproliferation.⁽¹³⁾ DGK α localizes in the cytoplasm and translocates to the plasma membrane in response to external stimuli.⁽¹⁴⁾

Because no specific anti-DGK α monoclonal antibody (mAb) was available to detect human DGK α by immunostaining, the localization of the native protein was not clarified. Recently, we reported a novel antihuman DGK α mAb, DaMab-2 (mouse IgG₁, 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 α cDNA⁽¹⁵⁾ was synthesized and subcloned into an expression vector, pMAL-c2 (New England Biolabs, Inc., Beverly, MA), with PA tag (GVAMPGAEDDVV),⁽¹⁶⁾ using the In-Fusion HD Cloning kit (Takara Bio, Inc., Shiga, Japan), named pMAL-c2-DGK α -PA. The deletion mutants of DGK α 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 α amino acids 240–260 with either alanine or glycine in dC320 of DGK α 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⁽¹⁷⁾ 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⁽¹⁸⁾ 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. EPITOPE 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	YTVHDQCQGMKALPCEVSTYAK	+	+
M248A	YTVHDQCAAKALPCEVSTYAK	+	+
K249A	YTVHDQCAMAALPCEVSTYAK	–	+
A250G	YTVHDQCAMKGLPCEVSTYAK	+	+
L251A	YTVHDQCAMKAAPCEVSTYAK	+	+
P252A	YTVHDQCAMKALACEVSTYAK	–	+
C253A	YTVHDQCAMKALPAEVSTYAK	–	+
E254A	YTVHDQCAMKALPCAVSTYAK	+	+
V255A	YTVHDQCAMKALPCEASTYAK	+	+
S256A	YTVHDQCAMKALPCEVATYAK	+	+
T257A	YTVHDQCAMKALPCEVSAYAK	+	+
Y258A	YTVHDQCAMKALPCEVSTAAG	+	+
A259G	YTVHDQCAMKALPCEVSTYGG	+	+
K260A	YTVHDQCAMKALPCEVSTYAA	+	+

Italic letters show the substitution of amino acid.

FIG. 2. Epitope mapping of DaMab-2 using deletion and point mutants of DGK α . (A) Immunoprecipitates of point mutants were electrophoresed and transferred onto PVDF membranes. After blocking, membranes were incubated with 20 μ g/mL DaMab-2 or 1 μ 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 α and developed a clone (DaMab-2).⁽¹⁷⁾ DaMab-2 recognized only DGK α in enzyme-linked immunosorbent assay and did not react with other isozymes, such as DGK γ , DGK ζ , DGK η , and DGK δ . 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 α (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 α 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 α protein. Our findings can be applied for the production of more functional anti-DGK α mAbs.

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Address correspondence to:

Yukinari Kato
 Department of Antibody Drug Development
 Tohoku University Graduate School of Medicine
 New Industry Creation Hatchery Center
 Tohoku University
 2-1, Seiryomachi, Aoba-ku
 Sendai 980-8575
 Japan

E-mail: yukinarikato@med.tohoku.ac.jp

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