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Original Articles

Epitope Mapping of Antidiacylglycerol Kinase α 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. DGKα, one of the 10 DGK isozymes, is involved in T cell function and has been shown to localize in the cytoplasm and nucleus. Furthermore, DGKα translocates to the plasma membrane in response to T cell receptor stimulation. Recently, we developed a specific monoclonal antibody (mAb), DaMab-2 (mouse IgG_1, kappa), against DGKα. 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 DGKα are important for DaMab-2 binding to the DGKα protein. Our findings can be applied for the production of more functional anti-DGKα mAbs.

Keywords: diacylglycerol kinase, DGKα, 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-1α, atypical PKCζ, 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. DGKα is the first identified enzyme of 80 kDa that contains an EF-hand motif (Ca^{2+}-binding site), a Zn finger (C1 domain, DG-binding site), and a catalytic domain. Various cell types and tissues express DGKα 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] DGKα 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 DGKα-deficient mice show an altered activity of TCR signaling and hyperproliferation.[13] DGKα localizes in the cytoplasm and translocates to the plasma membrane in response to external stimuli.[14]

Because no specific anti-DGKα monoclonal antibody (mAb) was available to detect human DGKα by immunostaining, the localization of the native protein was not clarified. Recently, we reported a novel antihuman DGKα mAb, DaMab-2 (mouse IgG_1, 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 DGKα 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-DGKα-PA. The deletion mutants of DGKα 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 DGKα amino acids 240–260 with either alanine or glycine in dC320 of DGKα was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa

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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 overexpressed DGKα has been usually used in these studies.

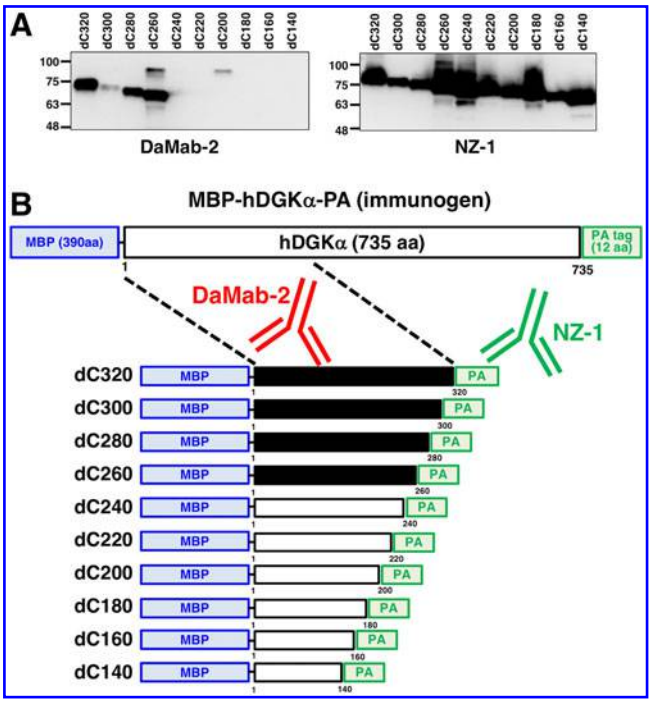

FIG. 1. Production of DGKα deletion mutants. **(A)** Immunoprecipitates of deletion mutants were electrophoresed and transferred onto PVDF membranes. After blocking, membranes were incubated with 20 μg/mL DaMab-2 or 1 μg/mL anti-PA tag (NZ-1) followed by peroxidase-conjugated antimouse or antirat IgG. **(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	*A*TVHDQCAMKALPCEVSTYAK	+	+
T241A	Y*A*VHDQCAMKALPCEVSTYAK	+	+
V242A	YT*A*HDQCAMKALPCEVSTYAK	+	+
H243A	YTV*A*DQCAMKALPCEVSTYAK	+	+
D244A	YTVH*A*QCAMKALPCEVSTYAK	+	+
Q245A	YTVHD*A*CAMKALPCEVSTYAK	+	+
C246A	YTVHDQ*A*AMKALPCEVSTYAK	−	+
A247G	YTVHDQC*G*MKALPCEVSTYAK	+	+
M248A	YTVHDQCA*A*KALPCEVSTYAK	+	+
K249A	YTVHDQCAM*A*ALPCEVSTYAK	−	+
A250G	YTVHDQCAMK*G*LPCEVSTYAK	+	+
L251A	YTVHDQCAMKA*A*PCEVSTYAK	+	+
P252A	YTVHDQCAMKAL*A*CEVSTYAK	−	+
C253A	YTVHDQCAMKALP*A*EVSTYAK	−	+
E254A	YTVHDQCAMKALPC*A*VSTYAK	+	+
V255A	YTVHDQCAMKALPCE*A*STYAK	+	+
S256A	YTVHDQCAMKALPCEV*A*TYAK	+	+
T257A	YTVHDQCAMKALPCEVS*A*YAK	+	+
Y258A	YTVHDQCAMKALPCEVST*A*AK	+	+
A259G	YTVHDQCAMKALPCEVSTY*G*K	+	+
K260A	YTVHDQCAMKALPCEVSTYA*A*	+	+

Italic letters show the substitution of amino acid.

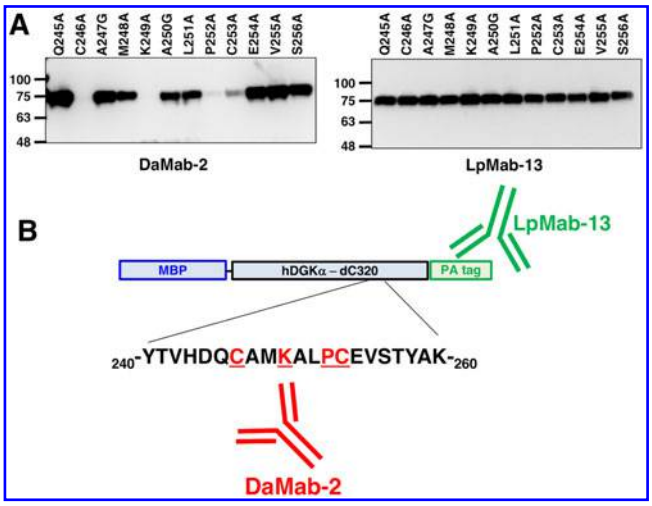

FIG. 2. Epitope mapping of DaMab-2 using deletion and point mutants of DGKα. **(A)** Immunoprecipitates of point mutants were electrophoresed and transferred onto PVDF membranes. After blocking, membranes were incubated with 20 μg/mL DaMab-2 or 1 μg/mL anti-PA tag (LpMab-13) followed by peroxidase-conjugated 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 DGKα and developed a clone (DaMab-2).[17] DaMab-2 recognized only DGKα in enzyme-linked immunosorbent assay and did not react with other isozymes, such as DGKγ, DGKζ, DGKη, and DGKδ. 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 DGKα (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 DGKα 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 DGKα protein. Our findings can be applied for the production of more functional anti-DGKα mAbs.

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Author Disclosure Statement

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