

Epitope Mapping of Antihuman Diacylglycerol Kinase Gamma Monoclonal Antibody DgMab-6

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Diacylglycerol kinase (DGK) converts diacylglycerol (DG) into phosphatidic acid (PA). Both DG and PA serve as lipidic second messengers; therefore, DGK plays a critical role in regulating the balance of two signaling pathways mediated by DG and PA in cellular functions. DGK gamma (DGK γ), 1 of the 10 mammalian DGK isozymes, has been reported to be involved in membrane traffic, mast cell function, and leukemic cell differentiation. We previously developed a novel anti-DGK γ monoclonal antibody (mAb), DgMab-6, which is very useful in immunocytochemistry of human cultured cells. In this study, we characterized the binding epitope of DgMab-6 using Western blotting, and Glu12 is important for facilitating the DgMab-6 binding to the DGK γ protein. These results might lead to further development for sensitive and specific mAbs against DGK γ .

Keywords: diacylglycerol kinase, DGK γ , monoclonal antibody, DgMab-6

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 it 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 functions as a messenger molecule that activates the hypoxia-inducible factor-1 α , atypical PKC ζ , and mammalian target of rapamycin. DGK constitutes an enzyme family comprising 10 isozymes of the mammalian species.^(1,2) Each isozyme has a distinct molecular structure and subcellular localization pattern. DGK gamma (DGK γ) was first isolated in rat⁽⁴⁾ and human,⁽⁵⁾ and is classified into type I DGK, which contains two EF hand motifs (Ca²⁺-binding site), two Zn fingers (DG-binding C1 domain), and a catalytic domain. DGK γ has been reported to be involved in a variety of cell functions including antigen-induced mast cell degranulation,⁽⁶⁾ leukemic cell differentiation,⁽⁷⁾ and membrane traffic between the endoplasmic reticulum and Golgi apparatus.⁽⁸⁾

We previously reported a novel antihuman DGK γ monoclonal antibody (mAb), DgMab-6, which is very useful in immunocytochemical analysis of cultured cells. In this study, we characterize the binding epitope of DgMab-6 using Western blotting.

Materials and Methods

Plasmid of DGK γ

Human DGK γ cDNA was synthesized, and was subcloned into an expression vector, pMAL-c2 (New England Biolabs, Inc., Beverly, MA), with PA tag (GVAMPGAEDDVV)⁽⁹⁾ using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan); the resultant construct was named pMAL-c2-DGK γ -PA. The deletion mutants of DGK γ produced using PCR were subcloned into pMAL-c2 with PA tag using the In-Fusion HD Cloning Kit. The substitution of DGK γ amino acids (aas) 6–20 with alanine in dC200 of DGK γ was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA). These constructs were verified using direct DNA sequencing.

Western blotting

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc., Waltham, MA) were transformed and cultured overnight at 37°C in Luria–Bertani medium (Thermo Fisher Scientific, Inc.) containing 100 μ g/mL ampicillin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The cell pellets were resuspended in phosphate-buffered saline containing 1% Triton X-100 and 50 μ g/mL aprotinin (Sigma-Aldrich Corp., St. Louis, MO). Lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc., Kyoto, Japan). About dC80 lysates

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were immunoprecipitated using amylose resin (New England Biolabs, Inc.) and boiled in SDS sample buffer (Nacalai Tesque, Inc.). The samples were electrophoresed on 5%–20% polyacrylamide gels (Nacalai Tesque, Inc.) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) for 1 hour, the membrane was incubated with DgMab-6 for 1 hour, followed by peroxidase-conjugated antimouse IgG (1:2000 dilution; Agilent Technologies, Inc.) for 1 hour. The membrane was also incubated with NZ-1 (anti-PA tag)⁽¹⁰⁾ for 1 hour, followed by biotin-conjugated antirat IgG (1:1000 dilution; Agilent Technologies, Inc.) for 30 minutes, and further incubated with VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes. The membrane was finally developed with the ImmunoStar LD Chemiluminescence Reagent (FUJIFILM Wako Pure Chemical Corporation) using the Sayaca-Imager (DRC Co., Ltd., Tokyo, Japan). All procedures of Western blotting were performed at room temperature.

Results

We herein characterized the binding epitope of DgMab-6 using Western blotting. As shown in Figure 1, we produced 19 C-terminal deletion mutants of DGK γ (i.e., dC380, dC360, dC340, dN320, dC300, dC280, dC260, dC240, dC220, dC200, dC180, dC160, dC140, dC120, dC100, dC80,

dC60, dC40, and dC20). Western blotting demonstrated that DgMab-6 detected all deletion mutants, which were also detected by an anti-PA tag mAb, NZ-1 (Fig. 1A), indicating that the N-terminus of the DgMab-6 epitope exists between 1st aa and 20th aa.

Western blotting of the additional deletion mutants of DGK γ —that is, dN6, dN11, dN16, and dN21—demonstrated that DgMab-6 detected dN6, but not dN11, dN16, and dN21, although all deletion mutants were detected by an anti-PA tag mAb, NZ-1 (Fig. 1B), thereby indicating that the N-terminus of the DgMab-6-epitope exists between 6th aa and 20th aa of DGK γ (Fig. 1B). These results are summarized in Figure 2.

Accordingly, we produced the following 15 DGK γ point mutants using human DGK γ -dC200: W6A, V7A, S8A, L9A, T10A, P11A, E12A, E13A, F14A, D15A, Q16A, L17A, Q18A, K19A, and Y20A. Western blotting demonstrated that the anti-PA tag mAb, NZ-1, detected all point mutants (Fig. 3A). In contrast, DgMab-6 did not detect E12A. These results are summarized in Figure 3B.

Discussion

Immunohistochemical examination using anti-DGK γ antibodies on rat tissues demonstrated cytoplasmic localization of DGK γ in several neurons of the brain.^(4,11) In contrast, morphological examination on human cells or tissues has not yet been performed because highly specific and sensitive

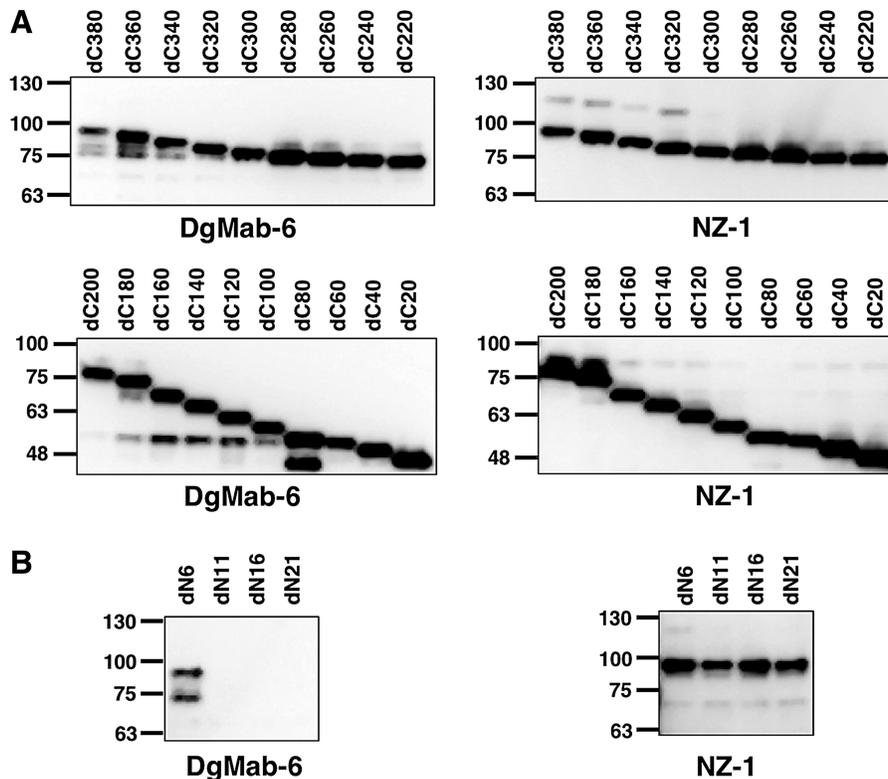


FIG. 1. Production of DGK γ deletion mutants. (A) C-terminal deletion mutants. (B) N-terminal deletion mutants. Cell lysates of deletion mutants or immunoprecipitates of dC80 were electrophoresed and transferred onto PVDF membranes. After blocking, the membranes were incubated with 1 μ g/mL of DgMab-6 or anti-PA tag (NZ-1), followed by incubation with peroxidase-conjugated antimouse or antirat IgG. DGK γ , diacylglycerol kinase gamma; PVDF, polyvinylidene difluoride.

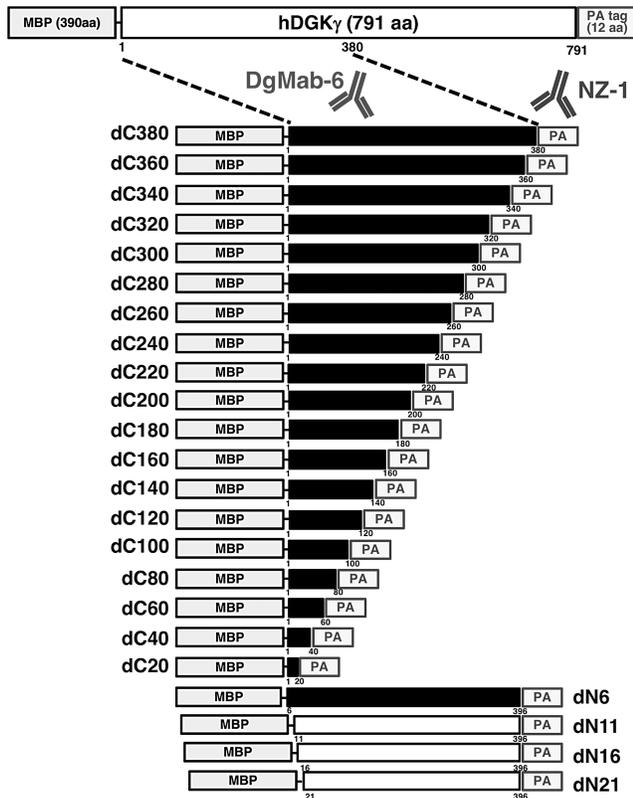


FIG. 2. Schematic illustration of DgMab-6 epitope. Black bars, deletion mutants detected by DgMab-6. hDGK γ , human DGK γ ; MBP, maltose-binding protein.

mAbs against human DGK γ had not been established. Recently, the recombinant MBP-DGK γ -PA was captured using NZ-1-Sepharose and efficiently eluted with PA tag peptide.⁽⁹⁾ Mice were then immunized with MBP-DGK γ -PA to develop novel anti-DGK γ mAbs. Using ELISA, the culture supernatants of hybridomas were screened for binding to recombinant MBP-DGK γ -PA. Finally, DgMab-6 (mouse IgG₁, kappa) was established. Immunocytochemical analysis revealed that immunoreactivity for DgMab-6 is detected as granular pattern in the cytoplasm in HL-60 cells, indicating that a novel anti-DGK γ mAb, DgMab-6, sensitively and specifically reacted with human DGK γ using immunocytochemical analysis. Previously, we further developed DaMab-2 (mouse IgG₁, kappa), a specific mAb against DGK α , which is extremely useful in immunocytochemical analysis.⁽¹²⁾ The binding epitopes of DaMab-2 were determined to be Cys246, Lys249, Pro252, and Cys253 of DGK α using Western blot analysis.⁽¹³⁾ Because many cysteine residues are included in DGK α (24 cysteins in 735 aas; 3.3%), we could not synthesize DGK α peptides, which easily aggregate. Of interest, DaMab-2 epitope was included in Zn finger, and two cysteine residues such as Cys246 and Cys253 are involved in the DaMab-2 epitope. In this study, using Western blotting, we also characterized the binding epitope of DgMab-6 for DGK γ . DgMab-6 did not detect E12A mutant (Fig. 3A), indicating that Glu12 is critical for DgMab-6 binding to DGK γ (Fig. 3B). This epitope exists in the N-terminal domain of DGK γ (Fig. 3C). These findings could be applied for the production of more functional anti-DGK γ mAbs.

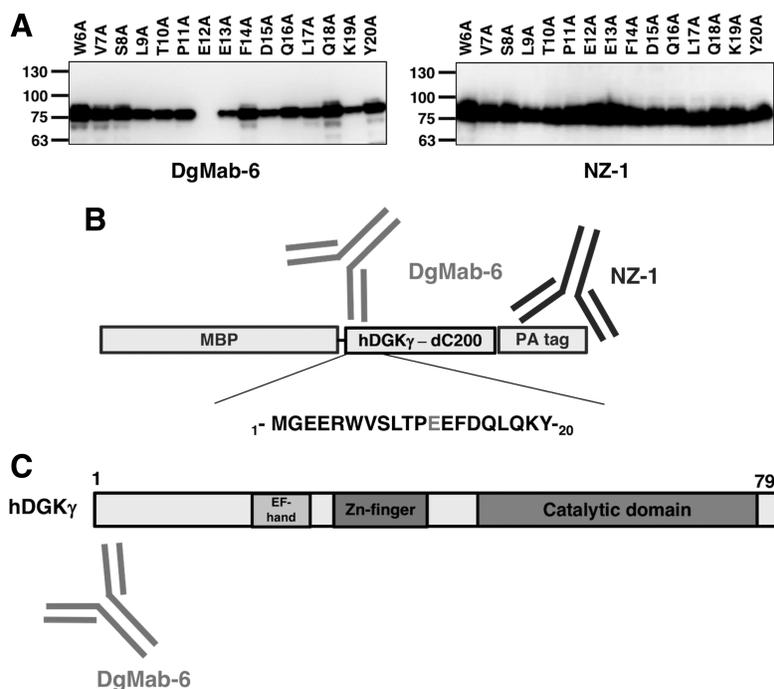


FIG. 3. Epitope mapping of DgMab-6 using point mutants of DGK γ . (A) Cell lysates of point mutants were electrophoresed and transferred onto PVDF membranes. After blocking, the membranes were incubated with 1 μ g/mL of DgMab-6 and anti-PA tag (NZ-1), followed by incubation with peroxidase-conjugated antimouse IgG and biotin-conjugated antirat IgG, respectively. (B) Schematic illustration of DgMab-6 epitope. A light gray amino acid is a critical epitope of DgMab-6. (C) Schematic illustration of DGK γ . The critical epitope of DgMab-6 is located in the N-terminus of DGK γ .

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