

Epitope Mapping of DhMab-1: An Antidiacylglycerol Kinase Monoclonal Antibody

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Diacylglycerol kinase (DGK) η is classified as a type II DGK and catalyzes diacylglycerol phosphorylation to produce phosphatidic acid. DGK η has been reported to be highly expressed in the hippocampus and cerebellum. Although a DGK η -specific monoclonal antibody (mAb) is necessary to reveal the association between the expression of DGK η and diseases, an anti-DGK η mAb for immunohistochemistry has not been developed. Recently, we established a specific antihuman DGK η (hDGK η) mAb, DhMab-1 (mouse IgG_{2a}, kappa). For epitope mapping of DhMab-1, here we produced deletion or point mutants of hDGK η and performed Western blotting to determine the binding epitope of DhMab-1. DhMab-1 reacted with the dN755 mutant, but not with the dN760 mutant, indicating that the N-terminus of the DhMab-1 epitope is mainly located between amino acids 755 and 760 of the protein. A more detailed analysis using point mutants demonstrated that seven mutants, that is, A751G, I755A, D756A, P757A, D758A, L759A, and D760A, were not detected by DhMab-1. These results indicate that Ala751, Ile755, Asp756, Pro757, Asp758, Leu759, and Asp760 are important for DhMab-1 binding to hDGK η .

Keywords: DGKh, DhMab-1, monoclonal antibody

Introduction

DIACYLGLYCEROL KINASE (DGK) plays a key role in the G-protein-mediated signaling pathway, which is an important signaling cascade in mammalian cells.^(1,2) DGK is an enzyme that phosphorylates diacylglycerol to produce phosphatidic acid. In turn, phosphatidic acid functions as a second messenger that mediates intracellular Ca²⁺ levels and regulates the mTOR-mediated signaling pathway.^(3,4) The DGK family consists of 10 isozymes in mammalian species, with each isozyme containing two to three C1 domains, while sharing a common catalytic domain.⁽¹⁾ The DGK family is further divided into five subtypes according to their subtype-specific functional domains.⁽⁵⁾ DGK η is classified as a type II DGK. It contains a pleckstrin homology (PH) domain and two C1 domains, a catalytic domain, and an accessory domain.⁽⁶⁾ The PH domain is a phosphatidylinositol-binding domain. In particular, the PH domain of DGK η binds to phosphatidylinositol 4,5-bisphosphate and regulates the subcellular localization of DGK η .⁽⁷⁾

Recently, we established a specific antihuman DGK η (hDGK η) monoclonal antibody (mAb), DhMab-1 (mouse IgG_{2a}, kappa). In this study, we performed epitope mapping to identify the binding epitope of DhMab-1 using deletion and point mutations of hDGK η .

Materials and Methods

Plasmid preparation and production of recombinant DGK η protein

Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding hDGK η (accession No. AB078967) plus a C-terminal PA tag (GVAMPGAEVV)^(8,9) was subcloned into the expression vector pMAL-c2 (New England Biolabs, Inc., Beverly, MA) using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan). The PA tag is recognized by an anti-PA tag mAb (NZ-1).⁽¹⁰⁾ The resulting construct was named pMAL-c2-hDGK η -PA. The deletion mutants of hDGK η were produced by polymerase chain reaction and were subcloned into pMAL-c2 with a PA tag using the In-Fusion HD Cloning Kit. The substitution of the amino acids of hDGK η with alanine at dN705 of hDGK η was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA). These constructs were verified by direct DNA sequencing.

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc., Waltham, MA) were transformed with the pMAL-c2-hDGK η -PA plasmid. They were cultured overnight at 37°C in Luria-Bertani broth (Thermo Fisher Scientific, Inc.) containing 100 µg/mL ampicillin (Sigma-Aldrich

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Corp., St. Louis, MO). Cell pellets were resuspended in phosphate-buffered saline containing 1% Triton X-100 and 50 µg/mL aprotinin (Sigma-Aldrich Corp.). After sonication, the crude extracts were collected by centrifugation (9000 g, 30 minutes, 4°C). The lysates were passed through a 0.45 µm 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 hours under gentle agitation. The resin was then transferred to a column and washed with 20 mL Tris-buffered saline (pH 7.5). The bound protein was eluted with the PA tag peptide at room temperature in a stepwise manner (1 mL × 10 washes).

Hybridoma production

The Animal Care and Use Committee of Tohoku University approved all animal experiments. DhMab-1 was produced using the mouse medial iliac lymph node method. In brief, B6D2F1/Slc mice (Japan SLC, Inc., Shizuoka, Japan) were immunized by injecting 33 µg of the pMAL-c2-hDGK η -PA protein together with Freund's complete adjuvant (Sigma-Aldrich Corp.) into their footpad. Additional immunization with 60 µg of the pMAL-c2-hDGK η -PA protein was performed through the tail base. The lymphocytes were fused with mouse myeloma Sp2/0-Ag14 cells using polyethylene glycol. The culture supernatants were screened using enzyme-linked immunosorbent assay of the binding to the pMAL-c2-hDGK η -PA protein.

Western blot analyses

Lysates were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc., Kyoto, Japan). The samples were electrophoresed on 5%–20% polyacrylamide gels (Nacalai Tesque, Inc.) and transferred onto a polyvinylidene difluoride membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) for 1 hour, the membrane was incubated with DhMab-1 (1 µg/mL) or NZ-1 (1 µg/mL) for 1 hour, followed by incubation with hydrogen peroxidase-conjugated antimouse immunoglobulins (1:2000 dilution; Agilent Technologies, Inc.) or HRP-conjugated antirat IgG (1:10,000 dilution; Sigma-Aldrich Corp.) for 1 hour. The membrane was developed with the ImmunoStar LD Chemiluminescence Reagent (FUJI-FILM Wako Pure Chemical Corporation) using the Sayaca-Imager (DRC Co., Ltd., Tokyo, Japan). All Western blot procedures were performed at room temperature.

Results

Previously, we established DhMab-1 (mouse IgG_{2a}, kappa), which can recognize hDGK η but not other related isozymes, such as DGK α , DGK γ , and DGK ζ , in an enzyme-linked immunosorbent assay (data not shown). In this study, we performed epitope mapping to characterize the binding epitope of DhMab-1 using Western blot analysis.

We produced a C-terminal deletion mutant (dC583) and an N-terminal deletion mutant (dN584), as shown in Figure 1. First, we investigated whether DhMab-1 recognizes either dC583 or dN584 by Western blotting. The results of this experiment indicated that DhMab-1 interacts with dN584 (Fig. 2A).

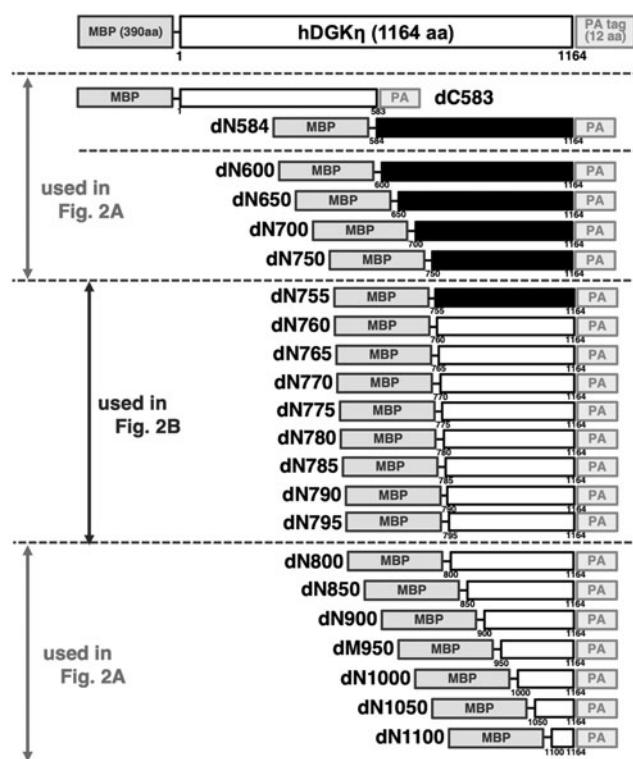


FIG. 1. Schematic illustration of the hDGK η deletion mutants. Black bars, deletion mutants detected by DhMab-1. DGK η , diacylglycerol kinase η ; hDGK η , human DGK η ; MBP, maltose-binding protein.

We then produced an additional 11 N-terminal deletion mutants (dN600, dN650, dN700, dN750, dN800, dN850, dN900, dN950, dN1000, dN1050, and dN1100) of hDGK η (Fig. 1) and performed Western blotting. As shown in Figure 2A, DhMab-1 recognized dN600, dN650, dN700, and dN750, but not dN800, dN850, dN900, dN950, dN1000, dN1050, and dN1100. All of the deletion mutants were detected by the anti-PA tag mAb, NZ-1 (Fig. 2A). This result indicates that the DhMab-1 epitope is located between amino acids 750 and 800.

Next, we produced an additional nine N-terminal deletion mutants (dN755, dN760, dN765, dN770, dN775, dN780, dN785, dN790, and dN795) of hDGK η (Fig. 1). Western blotting demonstrated that DhMab-1 recognized dN755, but not dN760, dN765, dN770, dN775, dN780, dN785, dN790, and dN795, indicating that the DhMab-1 epitope is located between amino acids 755 and 760. All of the deletion mutants were detected by the anti-PA tag mAb, NZ-1 (Fig. 2B).

To investigate further the critical epitope of DhMab-1, we produced constructs representing 16 point mutants within hDGK η (P748A, F749A, G750A, A751G, T752A, P753A, F754A, I755A, D756A, P757A, D758A, L759A, D760A, S761A, V762A, and D763A). All hDGK η point mutants were recognized by NZ-1 (Fig. 2C). In contrast, DhMab-1 did not recognize A751G, I755A, D756A, P757A, D758A, L759A, and D760A (Fig. 2C), indicating that DhMab-1 binds to DGK η through the following seven amino acids: Ala751, Ile755, Asp756, Pro757, Asp758, Leu759, and Asp760. These results are summarized in Figure 2D.

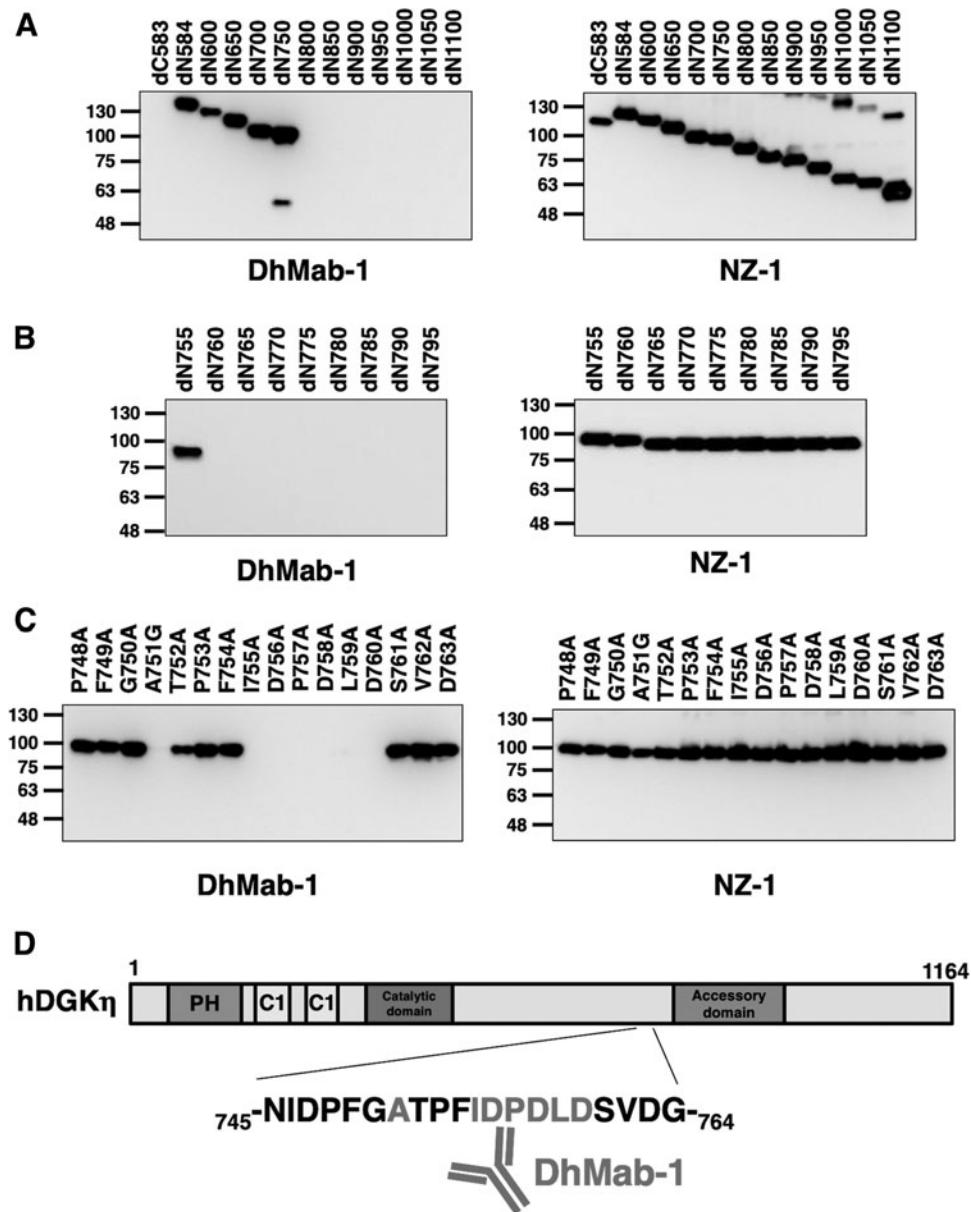


FIG. 2. Epitope mapping of DhMab-1 using deletion mutants and point mutants of hDGK η . (A) Cell lysates of the C- or N-terminal deletion mutants of hDGK η were electrophoresed and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μ g/mL of the DhMab-1 or anti-PA tag antibody (NZ-1). (B) Cell lysates of the N-terminal deletion mutants of hDGK η were electrophoresed and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μ g/mL of the DhMab-1 or anti-PA tag antibody (NZ-1). (C) Cell lysates of the dN705 point mutants of hDGK η were electrophoresed and transferred onto PVDF membranes. After blocking, the membranes were incubated with 1 μ g/mL of the DhMab-1 or anti-PA tag antibody (NZ-1). (D) Schematic illustration of hDGK η . The DhMab-1 epitope is located between the catalytic domain and the accessory domain. The amino acids shown in gray are important for DhMab-1 binding to hDGK η . PVDF, polyvinylidene difluoride.

Discussion

Previously, we established the anti-DGK α mAbs DaMab-2 and DaMab-8,^(11,12) an anti-DGK γ mAb, DgMab-6,⁽¹³⁾ and an anti-DGK ζ mAb, DzMab-1.⁽¹⁴⁾ We further determined their respective binding epitopes.^(12,15-17) DaMab-2 and DaMab-8 bind to the Zn-finger domain and catalytic domain of DGK α , respectively.^(12,15) DgMab-6 and DzMab-1 bind to the N-terminus of DGK γ and DGK ζ , respectively.^(16,17) Recently, we established a novel anti-hDGK η mAb, DhMab-1,

which is useful for immunohistochemical analysis (data not shown). In this study, we identified the binding epitope of DhMab-1 by Western blotting. Seven amino acids of hDGK η , that is, Ala751, Ile755, Asp756, Pro757, Asp758, Leu759, and Asp760, are critical for DhMab-1 binding to the hDGK η protein. This epitope is located near the accessory domain of hDGK η (Fig. 2D).

DGK η is highly expressed in the hippocampus and cerebellum areas of the brain.⁽¹⁸⁾ The DGK η mRNA is upregulated in patients with bipolar disorder.^(19,20) DGK η is also

highly expressed in lung cancer containing *EGFR* mutations, and knockdown of DGK η resulted in impaired growth of *EGFR*-mutant cell lines.⁽²¹⁾ DhMab-1 can be used to analyze the expression and pathophysiological function of DGK η and represents a useful tool to study the molecular basis of various diseases.

Author Disclosure Statement

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