Epitope mapping of an anti-diacylglycerol kinase delta monoclonal antibody DdMab-1

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ABSTRACT

Diacylglycerol kinase δ (DGKδ) is a type II DGK, which catalyzes diacylglycerol phosphorylation to produce phosphatidic acid. DGKδ is expressed in several types of tissues and organs including the stomach, testis, bone marrow, and lymph node. Here, we established an anti-human DGKδ (hDGKδ) mAb, DdMab-1 (mouse IgG2a, kappa), which is useful for Western blot analysis. We also introduced deletion or point mutations to hDGKδ, and performed western blotting to determine the binding epitope of DdMab-1. DdMab-1 reacted with the dN670 mutant, but not with the dN680 mutant, indicating that the N-terminus of the DdMab-1 epitope is mainly located between amino acids 670 and 680 of the protein. Further analysis using point mutants demonstrated that R675A, R678A, K679A, and K682A mutants were not detected, and V680A was only weakly detected by DdMab-1, indicating that Arg675, Arg678, Lys679, Val680 and Lys682 are important for binding of DdMab-1 to hDGKδ.

1. Introduction

Diacylglycerol kinase (DGK) plays a critical role in the regulation of numerous cellular functions by catalyzing the phosphorylation of diacylglycerol to phosphatidic acid [1,2]. Diacylglycerol activates protein kinase C, and the DGK terminates the diacylglycerol-mediated signaling pathway by phosphorylating diacylglycerol [3–7]. Here, the resulting phosphatidic acid functions as a second messenger which regulates the intracellular Ca2+ level and the mTOR-mediated signaling pathway [8,9].

Ten isozymes of the DGK family have been so far identified in mammals [2]. DGK family is also grouped into five subtypes based on their subtype-specific functional domains. DGKδ is one of the DGK family, and was first cloned from the human testis cDNA library [10].

DGKδ is expressed in several tissues and organs including the stomach, testis, bone marrow, and lymph node [11]. DGKδ is a type II DGK which contains pleckstrin homology (PH) and sterile alpha motif (SAM) domains at the N- and C-terminus of the protein, respectively. The PH domain can bind protein kinase C, the βγ-subunits of heterotrimeric G proteins, and phosphatidylinositol 4,5-bisphosphate [12–14]. On the other hand, the SAM domain has been shown to mediate both homo- and hetero-oligomerization, and therefore is a putative protein interaction module [15,16].

DGKδ was previously shown to regulate protein kinase C activity, and thereby control the degradation of epidermal growth factor receptor via modulation of ubiquitin-specific protease 8 expression in cultured human cells [17,18]. Moreover, DGKδ expression and activity levels are reduced in skeletal muscle tissues of Type 2 diabetic patients [19]. Hence, an anti-DGKδ monoclonal antibody (mAb) is required for specific detection of DGKδ in human tissues.

In this study, we established a novel anti-human DGKδ (hDGKδ) mAb, DdMab-1, by immunizing mice with recombinant hDGKδ. We also determined the binding epitope of DdMab-1 using deletion or point mutants of hDGKδ via Western blot analysis.

2. Materials and methods

2.1. Plasmid preparation

Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding hDGKδ (accession No.NM_152879) plus a C-terminal PA tag (GVAMP-GAEDDVV) [20] was subcloned into the pMAL-c2 expression vector.
plasmid. The cells were cultured overnight at 37 °C. The bacterial culture was transformed with the pMAL-c2-hDGK δ-PA plasmid. The bacteria were incubated with DdMab-1 (1 μg/mL or 10 μg/mL) or NZ-1 (1 μg/mL) for 1 h, then washed with 20 μL of Tris-buffered saline (TBS; pH 7.5). The bound protein was eluted with the PA tag peptide at room temperature in a stepwise manner (1 mL × 10 washes).

2.3. Hybridoma production

The Animal Care and Use Committee of Tohoku University approved all animal experiments. DdMab-1 was produced using the mouse medial iliac lymph node method. Briefly, three female 8-week old B6D2F1/Sldc mice (Japan SLC Inc., Shizuoka, Japan) were immunized by injecting 33 μg of the pMAL-c2-hDGK δ-PA protein and Freund's complete adjuvant (Sigma-Aldrich Corp.) into their footpad. Additional immunization with 50 μg of the pMAL-c2-hDGK δ-PA protein was performed via the tail base. The lymphocytes were fused with mouse myeloma Sp2/0-Ag14 cells using polyethylene glycol (PEG). The culture supernatants were screened using enzyme-linked immunosorbent assay for binding to the pMAL-c2-hDGK δ-PA protein. Because DdMab-1 reacted with an N-terminal deletion mutant (dN570) of hDGKδ, we then produced an additional 14 N-terminal deletion mutants (dN580, dN590, dN600, dN610, dN620, dN630, dN640, dN650, dN660, dN670, dN680, dN690, dN700, and dN710), and performed western blotting to detect the location of the epitope (Fig. 1A). As shown in Fig. 1B, DdMab-1 recognized dN580, dN590, dN600, dN610, dN620, dN630, dN640, dN650, dN660, dN670, dN680, dN690, dN700, and dN710 mutants. All of these deletion mutants were detected by the NZ-1 anti-PA tag antibody (Fig. 1B). Hence, the N-terminus of DdMab-1 epitope was found to be located between amino acids 670 and 680 of hDGKδ.

3. Results

3.1. Establishment of anti-hDGKδ mAbs

Three B6D2F1/Sldc mice were immunized by injecting 33 μg of the pMAL-c2-hDGK δ-PA protein into their footpad. Additional immunization with 50 μg of the pMAL-c2-hDGK δ-PA protein was performed via the tail base. The lymphocytes were fused with mouse myeloma Sp2/0-Ag14 cells using polyethylene glycol (PEG). The culture supernatants were screened using enzyme-linked immunosorbent assay for binding to the pMAL-c2-hDGK δ-PA protein. (B) Cell lysates of hDGKδ N-terminal deletion mutants were electrophoresed, and then transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of DdMab-1 or anti-PA tag antibody (NZ-1).

3.2. Epitope mapping of DdMab-1 using deletion mutant of hDGKδ

We also produced further hDGKδ constructs including 26 alanine point mutations to identify the critical DdMab-1 epitope (G670A, V671A, P672A, K673A, G674A, R675A, S676A, Q677A, R678A, K679A, V680A, S681A, K682A, S683A, P684A, C685A, E686A, K687A, L688A, etc.) and to identify the critical DdMab-1 epitope (G670A, V671A, P672A, K673A, G674A, R675A, S676A, Q677A, R678A, K679A, V680A, S681A, K682A, S683A, P684A, C685A, E686A, K687A, L688A, etc.).

3.3. Epitope mapping of DdMab-1 using point mutants of hDGKδ

Due to the critical DdMab-1 epitope (G670A, V671A, P672A, K673A, G674A, R675A, S676A, Q677A, R678A, K679A, V680A, S681A, K682A, S683A, P684A, C685A, E686A, K687A, L688A, etc.), we produced further hDGKδ constructs including 26 alanine point mutations to identify the critical DdMab-1 epitope (G670A, V671A, P672A, K673A, G674A, R675A, S676A, Q677A, R678A, K679A, V680A, S681A, K682A, S683A, P684A, C685A, E686A, K687A, L688A, etc.) and to identify the critical DdMab-1 epitope (G670A, V671A, P672A, K673A, G674A, R675A, S676A, Q677A, R678A, K679A, V680A, S681A, K682A, S683A, P684A, C685A, E686A, K687A, L688A, etc.) and to identify the critical DdMab-1 epitope (G670A, V671A, P672A, K673A, G674A, R675A, S676A, Q677A, R678A, K679A, V680A, S681A, K682A, S683A, P684A, C685A, E686A, K687A, L688A, etc.).
Lys682. These results are summarized in Fig. 2B. The identified DdMab did not recognize R675A, R678A, K679A, and K682A mutants, and only mAb for use in immunocytochemistry or immunohistochemistry against 4. Discussion

DdMab-1 binds to DGK I689A, S690A, K691A, G692A, S693A, L694A, and S695A). All hDGK with 10 μg/ml of anti-PA tag antibody (NZ-1). (B) Schematic illustration of DdMab-1 epitope mapping. Underlined amino acids (Arg675, Arg678, Lys679, Val680 and Lys682) are important for binding of DdMab-1 to hDGK. (C) Schematic illustration of the hDGK structure. DdMab-1 epitope is located in between catalytic and accessory domains. PH, pleckstrin homology; SAM, sterile alpha motif.

DgMab-6 and DzMab-1 were shown to bind to the N-termini of DGK δζα, respectively [29, 30]. DhMab-1/DhMab-4 [27] as anti-DGKn mAbs for immunohistochemistry. We determined their respective binding epitopes [25–30]. Accordingly, DaMab-2 and DaMab-8 was found to bind to the Zn-finger and catalytic domains of DGKα, respectively [25, 28]. DgMab-6 and DzMab-1 were shown to bind to the N-termini of DGKγ and DGKζ, respectively [29, 36]. DhMab-1/DhMab-4 epitope was found to be located near the accessory domain of hDGKβ [26, 27]. These epitope analyses revealed that each sensitive and specific mAb for use in immunocytochemistry or immunohistochemistry against different DGK isotypes included different epitope regions.

Here, we reported a novel anti-hDGKβ mAb, DaMab-1, which is useful for Western blot analysis (Figs. 1B and 2A). We also identified the binding epitope of DaMab-1 by western blotting, and found Arg675, Arg678, Lys679, Val680 and Lys682 to be important for DaMab-1 binding to hDGKβ. The epitope of DaMab-1 is located between catalytic and accessory domains (Fig. 2C). In our next study, we will investigate the utility of this mAb in immunocytochemistry and immunohistochemistry analyses for detection of hDGK proteins in different tissues/organs including the stomach, testis, bone marrow, and lymph node.

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Fig. 2. Epitope mapping of DaMab-1 using point mutants of hDGKβ. (A) Cell lysates of point mutants of ΔDGKα were electrophoresed, and then transferred onto PVDF membranes. After blocking, the membranes were incubated with 10 μg/ml of DaMab-1 or 1 μg/ml of anti-PA tag antibody (NZ-1). (B) Schematic illustration of DaMab-1 epitope mapping. Underlined amino acids (Arg675, Arg678, Lys679, Val680 and Lys682) are important for binding of DaMab-1 to hDGKβ. (C) Schematic illustration of the hDGK structure. DaMab-1 epitope is located in between catalytic and accessory domains. PH, pleckstrin homology; SAM, sterile alpha motif.

I689A, S690A, K691A, G692A, S693A, L694A, and S695A). All hDGK point mutants were recognized by NZ-1 (Fig. 2A). In contrast, DaMab-1 did not recognize R675A, R678A, K679A, and K682A mutants, and only weakly reacted with the V680A mutant (Fig. 2A), indicating that DaMab-1 binds to DGKβ via Arg675, Arg678, Lys679, Val680 and Lys682. These results are summarized in Fig. 2B. The identified DaMab-1 epitope is located between the catalytic and accessory domains (Fig. 2C).

4. Discussion

Previously, we established DaMab-2 as an anti-DGKα mAb [22], DgMab-6 as an anti-DGKα mAb [23], and DzMab-1 as an anti-DGKζ mAb [24] for immunocytochemistry. We further developed DaMab-8 as an anti-DGKζ mAb [25] and DhMab-1 [26]/DhMab-4 [27] as anti-DGKn mAbs for immunohistochemistry. We determined their respective binding epitopes [25–30]. Accordingly, DaMab-2 and DaMab-8 was found to bind to the Zn-finger and catalytic domains of DGKα, respectively [25, 28]. DgMab-6 and DzMab-1 were shown to bind to the N-termini of DGKγ and DGKζ, respectively [29, 36]. DhMab-1/DhMab-4 epitope was found to be located near the accessory domain of hDGKβ [26, 27]. These epitope analyses revealed that each sensitive and specific mAb for use in immunocytochemistry or immunohistochemistry against different DGK isotypes included different epitope regions.

Here, we reported a novel anti-hDGKβ mAb, DaMab-1, which is useful for Western blot analysis (Figs. 1B and 2A). We also identified the binding epitope of DaMab-1 by western blotting, and found Arg675, Arg678, Lys679, Val680 and Lys682 to be important for DaMab-1 binding to hDGKβ. The epitope of DaMab-1 is located between catalytic and accessory domains (Fig. 2C). In our next study, we will investigate the utility of this mAb in immunocytochemistry and immunohistochemistry analyses for detection of hDGK proteins in different tissues/organs including the stomach, testis, bone marrow, and lymph node.

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