Epitope Mapping of the Anti-Diacylglycerol Kinase Monoclonal Antibody DhMab-4 for Immunohistochemical Analysis

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Diacylglycerol kinase (DGK) plays a pivotal role in intracellular signaling pathways in mammals. Activated G protein-coupled receptor activates phospholipase C (PLC) through heterotrimeric G protein, following which PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). DGK catalyzes DG phosphorylation to produce phosphatidic acid. DG and phosphatidic acid function as second messengers and their intracellular concentrations are regulated by DGK; therefore, DGK plays an important role in regulating many biological processes. There are ten DGK isozymes, of which DGKη is classified as a type II DGK. Reports have shown that DGKη is associated with several diseases; for example, it is highly expressed in the hippocampus and cerebellum and is a key element in bipolar disorder. Although a DGKη-specific monoclonal antibody (mAb) is necessary to reveal the association between the expression of DGKη and diseases, an anti-DGKη mAb for immunohistochemistry has not yet been established. In this study, we established a specific anti-human DGKη (hDGKη) mAb, DhMab-4 (mouse IgG_{2b}, kappa). DhMab-4 strongly stained Purkinje cells of human cerebellum in immunohistochemistry analysis. For epitope mapping of DhMab-4, we produced deletion or point mutants of hDGKη and performed western blotting to determine the binding epitope of DhMab-4. DhMab-4 reacted with dN745 mutant but not with dN750 mutant, indicating that the N-terminus of the DhMab-4 epitope is located between amino acids 745 and 750. More detailed analysis using point mutants demonstrated that five mutants, that is, D747A, P748A, F749A, G750A, and T752A, were not detected by DhMab-4. These results indicate that Asp747, Pro748, Phe749, Gly750, and Thr752 are important for DhMab-4 binding to hDGKη.

Keywords: DGKh, DhMab-4, 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] Activated G protein-coupled receptor catalyzes a guanine nucleotide exchange on the alpha subunit of the heterotrimeric G protein and resulting in its activation. An activated G protein subsequently activates phospholipase C (PLC).[3,4] PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3), which functions as a second messenger. IP_3 diffuses into the cytosol and increases Ca^{2+} concentration in the cytoplasm, whereas DG remains on the plasma membrane and activates protein kinase C (PKC).[5–8] DGK is an enzyme that phosphorylates DG and produces phosphatidic acid.[1,2] Phosphatidic acid also functions as a second messenger, which

mediates intracellular Ca^{2+} levels and regulates the mTOR-mediated signaling pathway.[9,10] Therefore, DGK regulates two signaling pathways to control a variety of biological functions.[11]

The DGK family consists of 10 isozymes in mammalian species and each isozyme contains 2 to 3 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.[12] DGKη was first cloned from hamster and is classified as a type II DGK. It exhibits pleckstrin homology (PH) domain and contains two C1 domains, a catalytic domain and an accessory domain.[13] It is known that the PH domain is a phosphatidylinositol binding domain. The PH domain of DGKη binds to PIP_2 strongly and highly selectively, thereby regulating the subcellular localization of DGKη.[14] There are two splicing variants of DGKη, DGKη1, and DGKη2. DGKη2 has a sterile alpha

motif domain at its carboxyl terminus, while the DGKη1 variant is devoid of this domain.[15]

In this study, we immunized mice with a recombinant human DGKη (hDGKη) protein and established a novel anti-hDGKη monoclonal antibody (mAb), DhMab-4, which may be used for immunohistochemistry. We also performed epitope mapping to reveal the binding epitope of DhMab-4 using deletion and point mutations of hDGKη.

Materials and Methods

Plasmid preparation

Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding hDGKη (Accession No.: AB078967) plus a C-terminal PA tag (GVAMPGAEDDVV),[16,17] which is recognized by an anti-PA tag mAb (NZ-1),[18] 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 resultant construct was named pMAL-c2-hDGKη-PA. The deletion mutants of hDGKη were produced by PCR and subcloned into pMAL-c2 with a PA tag using the In-Fusion HD Cloning Kit. The substitution of hDGKη amino acids with alanine at dN705 of hDGKη was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA). These constructs were verified by direct DNA sequencing.

Production of recombinant DGKη protein

Competent *Escherichia coli* TOP-10 cells (Thermo Fisher Scientific, Inc., Waltham, MA) were transformed with the plasmid pMAL-c2-hDGKη-PA. They were then cultured overnight at 37°C in the LB medium (Thermo Fisher Scientific, Inc.) containing 100 μg/mL ampicillin (Sigma-Aldrich Corp., St. Louis, MO). Cell pellets were resuspended in phosphate-buffered saline (PBS) with 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 (TBS) (pH 7.5). The bound protein was eluted with the PA tag peptide at room temperature in a stepwise manner (1 mL × 10).

Hybridoma production

DhMab-4 was produced using the mouse medial iliac lymph node method. The Animal Care and Use Committee of Tohoku University approved all of the animal experiments. Briefly, B6D2F1/Slc mice (Japan SLC, Inc., Shizuoka, Japan) were immunized by injecting 33 μg of pMAL-c2-hDGKη-PA protein, along with Freund's complete adjuvant (Sigma-Aldrich Corp.), into the footpad. Additional immunization with 60 μg of pMAL-c2-hDGKη-PA protein was performed using the tailbase. 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 pMAL-c2-hDGKη-PA protein.

Immunohistochemical analyses

Normal human cerebellum tissue was purchased from ProteoGenex, Inc. (Inglewood, CA). Histological sections of 4 μm thickness were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), the sections were incubated with DhMab-4 (5 μg/mL) for 1 hour at room temperature and treated using the EnVision+ Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed with hematoxylin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

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
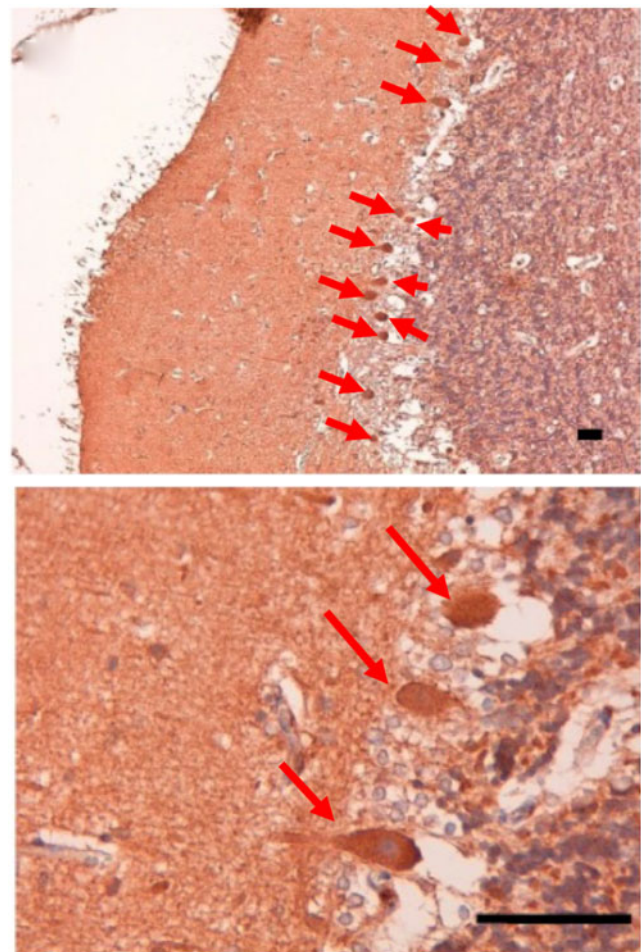

FIG. 1. Immunohistochemical analysis using DhMab-4 against human cerebellum. Tissue sections were incubated with DhMab-4 (5 μg/mL) for 1 hour at room temperature and treated using the EnVision+ kit for 30 minutes. Red arrows indicate Purkinje cells. Scale bar = 100 μm.

difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.) for 1 hour, the membrane was incubated with DhMab-4 or NZ-1 for 1 hour, followed by HRP-conjugated anti-mouse immunoglobulins (1:2000 dilution; Agilent Technologies, Inc.) or HRP-conjugated anti-rat IgG (1:10,000 dilution; Sigma-Aldrich Corp.) for 1 hour. The membrane was developed with the ImmunoStar LD Chemiluminescence Reagent (FUJIFILM Wako Pure Chemical Corporation) using the Sayaca-Imager (DRC Co., Ltd., Tokyo, Japan). All western blot procedures were performed at room temperature.

Results

In this study, we established DhMab-4 (mouse IgG$_{2b}$, kappa), which can recognize hDGKη, but not other related isozymes such as DGKα, DGKγ, and DGKζ in an enzyme-linked immunosorbent assay (data not shown). DhMab-4 stained human cerebral Purkinje cells as determined by immunohistochemistry (Fig. 1).

We next performed epitope mapping to characterize the binding epitope of DhMab-4 using western blot analysis.

First, we produced a C-terminal deletion mutant (dC583) and an N-terminal deletion mutant (dN584) and investigated whether DhMab-4 recognizes either dC583 or dN584 by western blot analysis. The results indicated that DhMab-4 interacts with dN584 (Fig. 2A, left panel). We then produced additional 11 N-terminal deletion mutants (dN600, dN650, dN700, dN750, dN800, dN850, dN900, dN950, dN1000, dN1050, and dN1100) of hDGKη and performed a western blot. As shown in Figure 2A (left panel), DhMab-4 recognized dN600, dN650, and dN700 but not dN750, dN800, dN850, dN900, dN950, dN1000, and dN1050. All of the deletion mutants were detected by an anti-PA tag mAb, NZ-1 (Fig. 2A, right panel). This result indicates that the N-terminus of the DhMab-4 epitope exists between 700 and 750 amino acids (aa) (Fig. 2B).

We next produced an additional nine N-terminal deletion mutants of hDGKη (dN705, dN710, dN715, dN720, dN725, dN730, dN735, dN740, and dN745). The western blot results showed that DhMab-4 and NZ-1 detected all of the deletion mutants (Fig. 3A), indicating that the N-terminus of the DhMab-4 binding epitope resides between amino acids 745 and 750 (Fig. 3B).
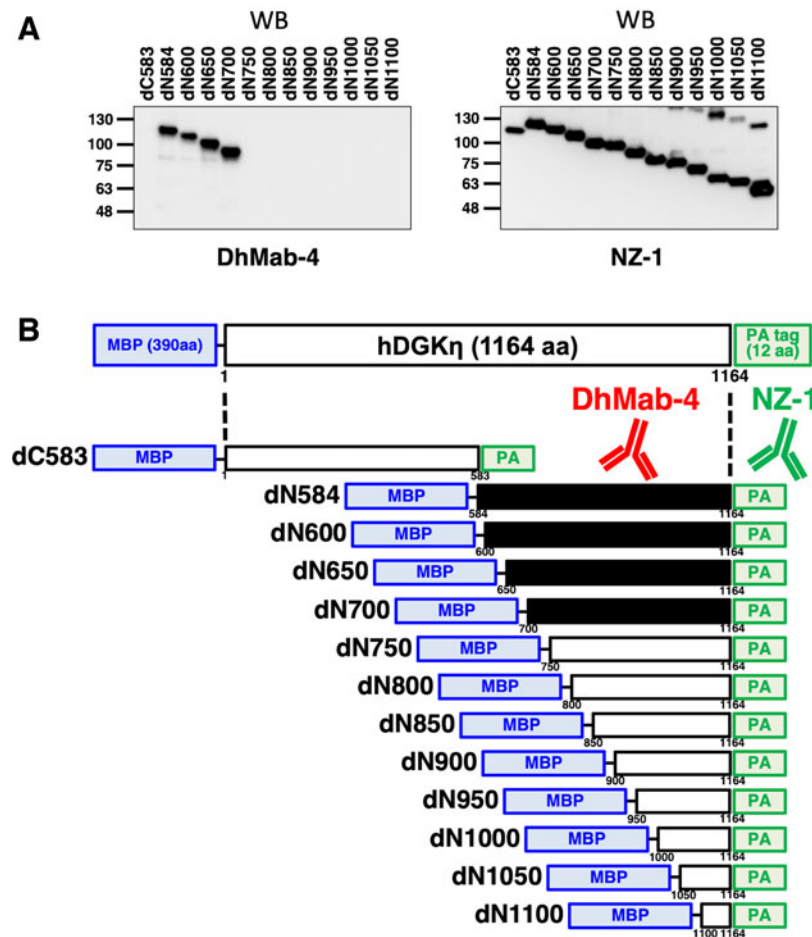

FIG. 2. Epitope mapping of DhMab-4 using deletion mutants of hDGKη. **(A)** Cell lysates of C- or N-terminal deletion mutants of hDGKη were electrophoresed and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of DhMab-4 or anti-PA tag antibody (NZ-1). **(B)** Schematic illustration of epitope mapping of DhMab-4. Black bars, deletion mutants detected by DhMab-4. DGK, diacylglycerol kinase; hDGKη, human DGKη; MBP, maltose-binding protein; PVDF, polyvinylidene difluoride.
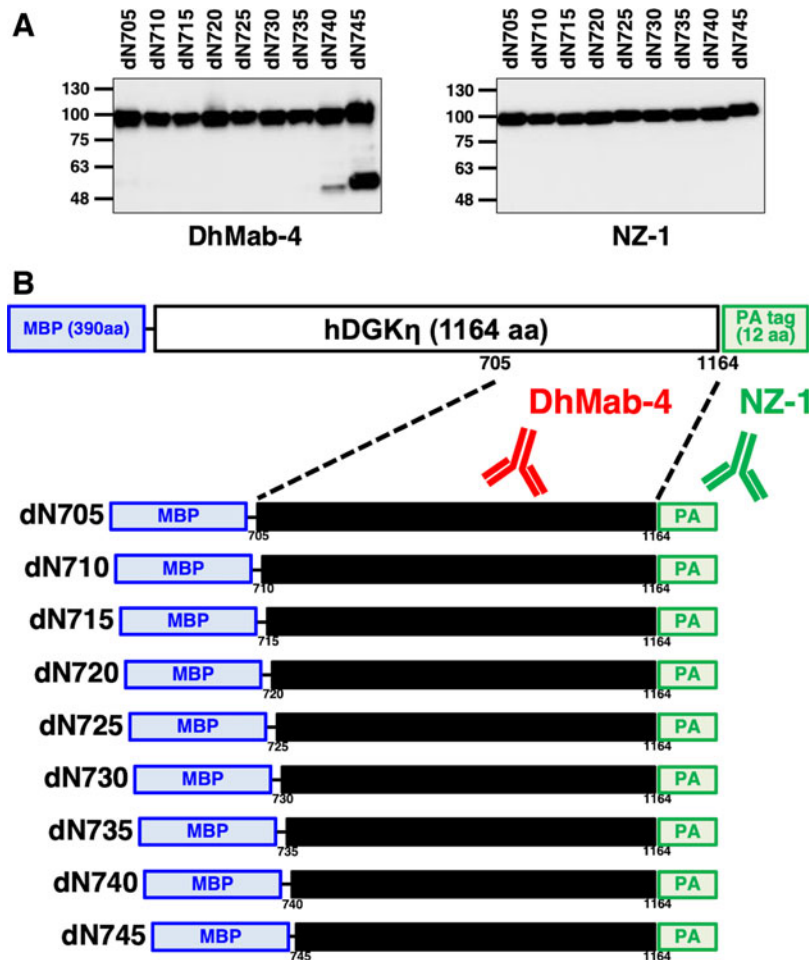
FIG. 3. Epitope mapping of DhMab-4 using N-terminal deletion mutants of hDGKη. **(A)** Cell lysates of N-terminal deletion mutants of hDGKη were electrophoresed and transferred onto a PVDF membrane. After blocking, the membrane was incubated with 1 μg/mL of DhMab-4 or anti-PA tag antibody (NZ-1). **(B)** Schematic illustration of epitope mapping of DhMab-4.

To further investigate the crucial epitope of DhMab-4, we produced constructs representing 15 point mutations within hDGKη (N745A, I746A, D747A, P748A, F749A, G750A, A751G, T752A, P753A, F754A, I755A, D756A, P757A, D758A, and L759A). All hDGKη point mutants were recognized by NZ-1 (Fig. 4A, right panel). In contrast, DhMab-4 did not recognize D747A, P748A, F749A, G750A, or T752A (Fig. 4A, left panel), indicating that DhMab-4 binds to DGKη through these five amino acids. These results are summarized in Figure 4B.

Discussion

DGK plays an important role in signaling pathways by regulating the intracellular level of DG and phosphatidic acid, and it is considered a potential therapeutic target for some diseases. Recently, we established anti-DGKα,[19,20] anti-DGKγ,[21] and anti-DGKζ[22] mAbs and determined their respective binding epitopes.[20,23–25] These mAbs are very useful for immunohistochemical analyses. However, we have not established anti-DGKη mAbs for immunohistochemistry.

In this study, we established a novel anti-hDGKη mAb, DhMab-4. DhMab-4 can detect hDGKη in formalin-fixed paraffin-embedded human cerebellum tissue, indicating that DhMab-4 sensitively interacts with hDGKη by immunohistochemical analysis (Fig. 1). We further demonstrated the binding epitope of DhMab-4 by western blot analyses. Five amino acids in hDGKη, Asp747, Pro748, Phe749, Gly750, and Thr752, are important for DhMab-4 binding to the hDGKη protein. This epitope is located near the accessory domain of hDGKη (Fig. 4C). The amino acid sequence of this region is highly conserved among vertebrate DGKη; hence, DhMab-4 may be useful for detecting DGKη in other species such as dog, chicken, lizard, and zebrafish.

DGKη is highly expressed in the brain, especially in the hippocampus and cerebellum.[26] The expression of DGKη mRNA is increased in patients with bipolar disorder.[27,28] Furthermore, behavioral tests using mice show that the behavioral profile is similar to human mania in DGKη knockout mice.[29] These results indicate that DGKη plays a key role in bipolar disorder. In addition, DGKη is also highly expressed in lung cancer containing EGFR mutations, and the knockdown of DGKη has resulted in impaired growth of EGFR
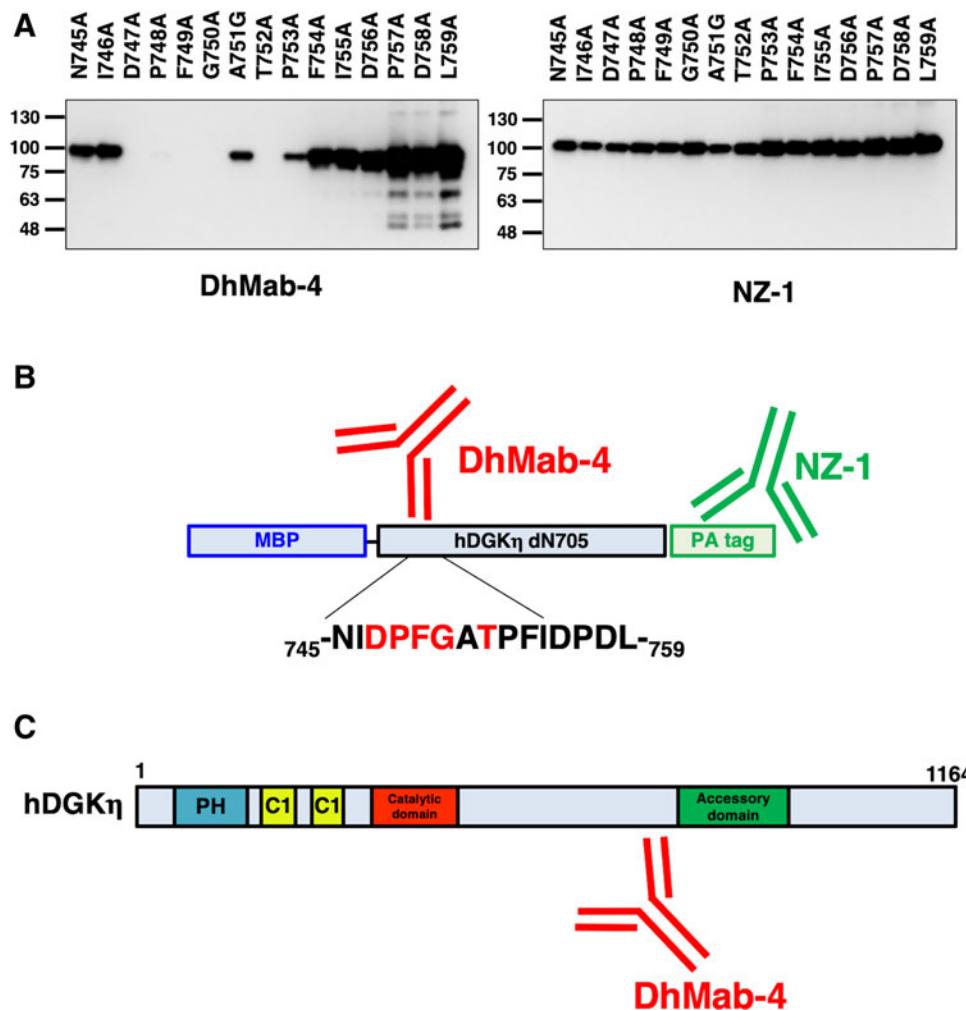
FIG. 4. Epitope mapping of DhMab-4 using hDGKη point mutants. **(A)** Cell lysates of point mutants of hDGKη dN705 were electrophoresed and transferred onto PVDF membranes. After blocking, the membranes were incubated with 1 μg/mL of DhMab-4 or anti-PA tag antibody (NZ-1). **(B)** Schematic illustration of epitope mapping of DhMab-4 and anti-PA tag antibody (NZ-1). Asp747, Pro748, Phe749, Gly750, and Thr752 are important for DhMab-4 binding to hDGKη. **(C)** Schematic illustration of hDGKη. DhMab-4 epitope is located in between catalytic domain and accessory domain.

mutant cell lines.[30] To understand the relationship between the expression of DGKη and these diseases by immunohistochemical methods, a DGKη-specific mAb has been required. DhMab-4 can be used to analyze the expression and pathophysiological function of DGKη and it represents a useful tool to study the molecular basis for various diseases.

Author Disclosure Statement

Funding Information

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