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Epitope Mapping of Anti-Diacylglycerol Kinase ζ Monoclonal Antibody for the Detection of T Cells by Immunohistochemical Analyses

Yukinari Kato,[1,2] Shunsuke Itai,[1] Shinji Yamada,[1] Hiroyoshi Suzuki,[3] and Mika K. Kaneko[1]

The diacylglycerol kinases (DGKs) are a family of proteins that catalyze the phosphorylation of the cell membrane lipid diacylglycerol (DG), a cellular component that is important in lipid biochemistry and signal transduction, into phosphatidic acid. DG-mediated signal transduction downstream of the T cell receptor has previously been reported to be terminated in most cases by one of 10 DGK isoforms, DGKζ. In this study, we performed immunohistochemical analysis using a rabbit anti-DGKζ monoclonal antibody (mAb) (clone EPR22040-80) against tissues from the tonsils of a patient with oropharyngeal squamous cell carcinoma. We demonstrated that many DGKζ-expressing T cells are localized in the tonsils. We further characterized the binding epitope using an enzyme-linked immunosorbent assay and found that Pro790, Gln791, Gly792, and Leu795 residues of DGKζ are important for facilitating anti-DGKζ mAb binding to DGKζ. This anti-DGKζ mAb could be valuable in immunohistochemical analyses in determining the distribution of DGKζ-expressing T cells in pathophysiological tissues.

Keywords: diacylglycerol kinase, DGKζ, monoclonal antibody, epitope mapping

Introduction

Diacylglycerol kinases (DGKs) are a family of proteins that phosphorylate the cell membrane lipid diacylglycerol (DG) into phosphatidic acid.[1–3] DG functions as an important second messenger in T cells.[4] DG-mediated signal transduction downstream of T cell receptors (TCRs) is terminated by DGKα and DGKζ, 2 of the 10 DGK isoforms.[5] DGKζ has been shown to be the dominant isoform.[6] T cells deficient in either DGKα or DGKζ are hyperresponsive, leading to enhanced proliferation and secretion of cytokines in response to TCR activation.[7–9] Riese et al. demonstrated that CD8+ T cells deficient in DGKs exhibit enhanced activity against xenografts after adoptive transfer of T cells when expressing TCRs or chimeric antigen receptors specific for tumor antigens.[10] Jing et al. reported that targeting DGKζ may increase the efficacy of adoptive T cell and immune checkpoint therapies in the treatment of leukemia.[11]

In the present study, we selected a commercially available rabbit anti-DGKζ monoclonal antibody (mAb) that is advantageous for immunohistochemical analysis and further characterized its binding epitope using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Immunohistochemical analyses

We used tissues from a patient with oropharyngeal squamous cell carcinoma who had undergone surgery at the Sendai Medical Center. Informed consent for sample procurement and subsequent data analyses was obtained from the patient or the patient's guardian. The tissue samples were processed to produce 4-μm paraffin-embedded tissue sections that were autoclaved in EnVision FLEX Target Retrieval Solution High pH (Agilent Technologies, Inc., Santa Clara, CA) for 20 minutes and then blocked using the SuperBlock T20 (PBS) blocking buffer (Thermo Fisher Scientific, Inc., Waltham, MA). Samples were incubated with rabbit anti-DGKζ mAb (clone: EPR22040-80; 1/4000 dilution; Abcam, Cambridge, United Kingdom) for 1 hour at room temperature and then treated using the Envision+ Kit for rabbit (Agilent Technologies, Inc.) for 30 minutes. The tissue sections were stained using 3,3′-

[1]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[2]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.
[3]Department of Pathology and Laboratory Medicine, Sendai Medical Center, Sendai, Japan.

diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 minutes and counterstained using hematoxylin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Enzyme-linked immunosorbent assay

The DGKζ peptides synthesized using PEPScreen (Sigma-Aldrich Corp., St. Louis, MO) were immobilized on Nunc MaxiSorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at concentrations of 10 μg/mL for 30 minutes at 37°C. Following blocking with SuperBlock T20 (PBS) blocking buffer, the plates were incubated with 1 μg/mL of rabbit anti-DGKζ mAb, followed by 1:1000 dilution of peroxidase-conjugated anti-rabbit IgG (Agilent Technologies, Inc.). The enzymatic reaction was performed using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA). These reactions were performed at 37°C using a total sample volume of 50–100 μL.

Inhibition assay

The 4-μm paraffin-embedded tissue sections were directly autoclaved in EnVision FLEX Target Retrieval Solution High pH (Agilent Technologies, Inc.) for 20 minutes and blocked using the SuperBlock T20 (PBS) blocking buffer (Thermo Fisher Scientific, Inc.), incubated with a rabbit anti-DGKζ mAb (clone: EPR22040-80; 1/4000 dilution; Abcam) plus peptides (1 μg/mL) for 1 hour at room temperature, and then treated using the Envision+ Kit for rabbit (Agilent Technologies, Inc.) for 30 minutes. The tissue sections were stained using DAB (Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Results

For immunohistochemical analysis, we used formalin-fixed and paraffin-embedded (FFPE) sections, including the tonsils of a patient with oropharyngeal squamous cell carcinoma, because DGKζ is known to be expressed in activated T cells.[7–9] As shown in Figure 1, the rabbit anti-DGKζ mAb

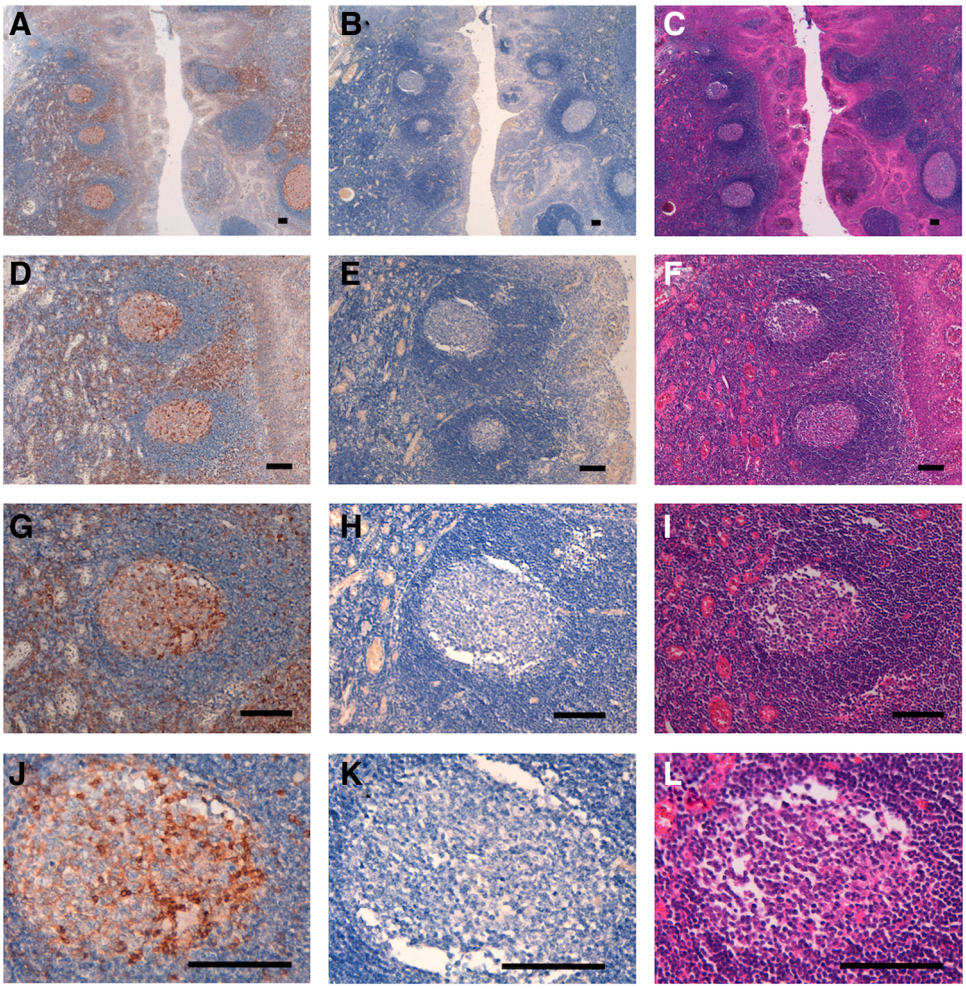

FIG. 1. Immunohistochemical analysis of DGKζ against oropharyngeal squamous cell carcinomas. Tissue sections were incubated with EPR22040-80 (1/4000 dilution; **A, D, G, J)** or blocking buffer **(B, E, H, K)** for 1 hour at room temperature and treated using the Envision+ Kit for rabbit (Agilent Technologies, Inc.) for 30 minutes. Scale bar = 100 μm. Hematoxylin and eosin staining **(C, F, I, L)**. DGK, diacylglycerol kinase.

Table 1. Determination of EPR22040-80 Epitope by Enzyme-Linked Immunosorbent Assay

Peptide	*Sequence*	*EPR22040-80*
1–20	MEPRDGSPEARSSDSESASA	—
11–30	RSSDSESASASSSGSERDAG	—
21–40	SSSGSERDAGPEPDKAPRRL	—
31–50	PEPDKAPRRLNKRRFPGLRL	—
41–60	NKRRFPGLRLFGHRKAITKS	—
51–70	FGHRKAITKSGLQHLAPPPP	—
61–80	GLQHLAPPPPTPGAPSSESE	—
71–90	TPGAPSSESERQIRSTVDWS	—
81–100	RQIRSTVDWSESATYGEHIW	—
91–110	ESATYGEHIWFETNVSGDFS	—
101–110	FETNVSGDFSYVGEQYSVAR	—
111–130	YVGEQYSVARMLQKSVSRRK	—
121–140	MLQKSVSRRKSAASKIVVHT	—
131–150	SAASKIVVHTPSIEQLEKIN	—
141–160	PSIEQLEKINFRSKPSFRES	—
151–170	FRSKPSFRESGSRNVREPTF	—
161–180	GSRNVREPTFVRHHWVHRRR	—
171–190	VRHHWVHRRRQDGKSRHSGK	—
181–200	QDGKSRHSGKGFQQKFTFHS	—
191–210	GFQQKFTFHSKEIVAISSSW	—
201–220	KEIVAISSSWSKQAYHSKVS	—
211–230	SKQAYHSKVSSFMLQQIEEP	—
221–240	SFMLQQIEEPSSLGVHAAVV	—
231–250	SSLGVHAAVVIPPTWILRAR	—
241–260	IPPTWILRARRPQNTLKASK	—
251–270	RPQNTLKASKKKKRASFKRK	—
261–280	KKKRASFKRKSSKKGPEEGR	—
271–290	SSKKGPEEGRWRPFIIRPTP	—
281–300	WRPFIIRPTPSPLMKPLLVF	—
291–310	SPLMKPLLVFVNPKSGGNQG	—
301–320	VNPKSGGNQGAKIIQSFLWY	—
311–330	AKIIQSFLWYLNPRQVFDLS	—
321–340	LNPRQVFDLSQGGPKEALEM	—
331–350	QGGPKEALEMYRKVHNLRIL	—
341–360	YRKVHNLRILASGGDGTVGW	—
351–370	ASGGDGTVGWILSTLDQLRL	—
361–380	ILSTLDQLRLKPPPVAILP	—
371–390	KPPPPVAILPLGTGNDLART	—
381–400	LGTGNDLARTLNWGGGYTDE	—
391–410	LNWGGGYTDEPVSKILSHVE	—
401–420	PVSKILSHVEEGNVVQLDRW	—
411–430	EGNVVQLDRWDLHAEPNPEA	—
421–440	DLHAEPNPEAGPEDRDEGAT	—
431–450	GPEDRDEGATDRLPLDVFNN	—
441–460	DRLPLDVFNNYFSLGFDAHV	—
451–470	YFSLGFDAHVTLEFHESREA	—
461–480	TLEFHESREANPEKFNSRFR	—
471–490	NPEKFNSRFRNKMFYAGTAF	—
481–500	NKMFYAGTAFSDFLMGSSKD	—
491–510	SDFLMGSSKDLAKHIRVVSD	—
501–520	LAKHIRVVSDGMDLTPKIQD	—
511–530	GMDLTPKIQDLKPQSVVFLN	—
521–540	LKPQSVVFLNIPRYSAGTMP	—
531–550	IPRYSAGTMPWGHPGEHHDF	—
541–560	WGHPGEHHDFEPQRHDDGYL	—
551–570	EPQRHDDGYLEVIGFTMTSL	—
561–580	EVIGFTMTSLAALQVGGHGE	—
571–590	AALQVGGHGERLTQSREVVL	—
581–600	RLTQSREVVLTTSKAIPVQV	—
591–610	TTSKAIPVQVDGEPSKLAAS	—
601–620	DGEPSKLAASRIRIALRNQA	—
611–630	RIRIALRNQATMVQKAKRRS	—
621–640	TMVQKAKRRSAAPLHSDQQP	—
631–650	AAPLHSDQQPVPEQLRIQVS	—
641–660	VPEQLRIQVSRVSMHDYEAL	—
651–670	RVSMHDYEALHYDKEQLKEA	—
661–680	HYDKEQLKEASVPLGTVVVP	—
671–690	SVPLGTVVVPGDSDLELSRA	—
681–700	GDSDLELSRAHIERLQQEPD	—
691–710	HIERLQQEPDGAGAKSPTSQ	—
701–720	GAGAKSPTSQKLSPKWSFLD	—
711–730	KLSPKWSFLDATTASRFYRI	—
721–740	ATTASRFYRIDRAQEHLNYV	—
731–750	DRAQEHLNYVTEIAQDEIYI	—
741–760	TEIAQDEIYILDPELLGASA	—
751–770	LDPELLGASARPDLPTPTSP	—
761–780	RPDLPTPTSPLPTSPSSPTP	—
771–790	LPTSPSSPTPRSLQGDAAPP	—
781–800	RSLQGDAAPPQGEELIEAAK	+++
791–810	QGEELIEAAKRNDFSKLQEL	—
801–820	RNDFSKLQELHRAGGDLMHR	—
811–830	HRAGGDLMHRDEQSRTLLHH	—
821–840	DEQSRTLLHHAVSTGSKDVV	—
831–850	AVSTGSKDVVRYLLDHAPPE	—
841–860	RYLLDHAPPEILDAVEENGE	—
851–870	ILDAVEENGETSLHQAAALG	—
861–880	TSLHQAAALGQRTISHYIVE	—
871–890	QRTISHYIVEAGASLMKTDQ	—
881–900	AGASLMKTDQQGDTPRQRAE	—
891–910	QGDTPRQRAEKAQDTELAAY	—
901–920	KAQDTELAAYLENRQHYQMI	—
911–929	LENRQHYQMIQREDQETAV	—

+++, OD655 ≧ 0.6; —, OD655 < 0.2.

Table 2. Determination of EPR22040-80 Epitope by Enzyme-Linked Immunosorbent Assay Using Point Mutants

Mutation	*Sequence*	*EPR22040-80*
R781A	**A**SLQGDAAPPQGEELIEAAK	+++
S782A	R**A**LQGDAAPPQGEELIEAAK	+++
L783A	RS**A**QGDAAPPQGEELIEAAK	+++
Q784A	RSL**A**GDAAPPQGEELIEAAK	+++
G785A	RSLQ**A**DAAPPQGEELIEAAK	+++
D786A	RSLQG**A**AAPPQGEELIEAAK	+++
A787G	RSLQGD**G**APPQGEELIEAAK	+++
A788G	RSLQGDA**G**PPQGEELIEAAK	+++
P789A	RSLQGDAA**A**PQGEELIEAAK	+++
P790A	RSLQGDAAP**A**QGEELIEAAK	++
Q791A	RSLQGDAAPP**A**GEELIEAAK	+
G792A	RSLQGDAAPPQ**A**EELIEAAK	—
E793A	RSLQGDAAPPQG**A**ELIEAAK	+++
E794A	RSLQGDAAPPQGE**A**LIEAAK	+++
L795A	RSLQGDAAPPQGEE**A**IEAAK	—
I796A	RSLQGDAAPPQGEEL**A**EAAK	+++
E797A	RSLQGDAAPPQGEELI**A**AAK	+++
A798G	RSLQGDAAPPQGEELIE**G**AK	+++
K799G	RSLQGDAAPPQGEELIEA**G**K	+++
K800A	RSLQGDAAPPQGEELIEAA**A**	+++

Mutated amino acids (Ala or Gly) are shown in bold letters.
+++, OD655 ≧ 0.6; ++, 0.4 ≦ OD655 < 0.6; +, 0.2 ≦ OD655 < 0.4; —, OD655 < 0.2.

(*continued*)

(clone: EPR22040-80) stained the T cells of the tonsils very strongly, indicating that EPR22040-80 is extremely useful for the detection of DGKζ in FFPE tissues.

Next, we examined the binding epitope of EPR22040-80. We first produced 92 synthetic peptides of DGKζ, in which all cysteine residues were converted into serine residues to avoid self-aggregation (Table 1). Using ELISA, we demonstrated that the 781–800 position peptide was detected by EPR22040-80. We also synthesized 20 peptides, including a number of point mutations of the peptide at positions 781–800 (Table 2). Almost all the point mutations were detected by EPR22040-80, except for P790A, Q791A, G792A, and L795A, indicating Pro790, Gln791, Gly792, and Leu795 are included in the critical epitope of EPR22040-80. The epitope of EPR22040-80 is summarized in Figure 2.

We further performed an inhibition assay using immunohistochemistry. Although EPR22040-80 reacted with the T cells of oropharyngeal squamous cell carcinoma, these reactions were completely neutralized by R781A. L795A did not block the reactions of EPR22040-80 (Fig. 3). These data supported the contention that Leu795 of DGKζ is critical for EPR22040-80 detection.

Discussion

We have developed anti-DGKα (clone: DaMab-2)[12] and anti-DGKγ (clone: DgMab-6)[13] mAbs, both of which are extremely useful for immunocytochemical analysis. We characterized the binding epitope of DaMab-2 using Western blots and revealed that the Cys246, Lys249, Pro252, and Cys253 residues of DGKα are important for binding of DaMab-2 to DGKα.[14] These findings could be applied for the production of more functional anti-DGKα mAbs. We have not developed anti-DGKζ mAbs, which are useful for immunocytochemical and immunohistochemical analyses. In particular, it is difficult to develop mAbs for immunohistochemical analyses against FFPE tissue sections. Therefore, we first characterized commercially available anti-DGKζ mAbs. Among several anti-DGKζ mAbs, a rabbit anti-DGKζ mAb (clone: EPR22040-80 from Abcam) stained the T cells of tonsils very strongly. According to the Abcam product datasheet, EPR22040-80 is produced by immunizing recombinant fragments within human DGKζ (700 aa–1000 aa). However, the critical epitope of EPR22040-80 has not been determined. In this study, ELISA demonstrated that Pro790, Gln791, Gly792, and Leu795 of DGKζ are included in the critical epitope of EPR22040-80. This epitope seems to be included between catalytic domain and ankyrin repeats of DGKζ (Fig. 2). EPR22040-80 could be valuable for immunohistochemical analyses and in clarifying our understanding of the distribution of DGKζ-expressing T cells in a wide range of pathophysiological tissues. These findings can be applied to the production of more functional anti-DGKζ mAbs.

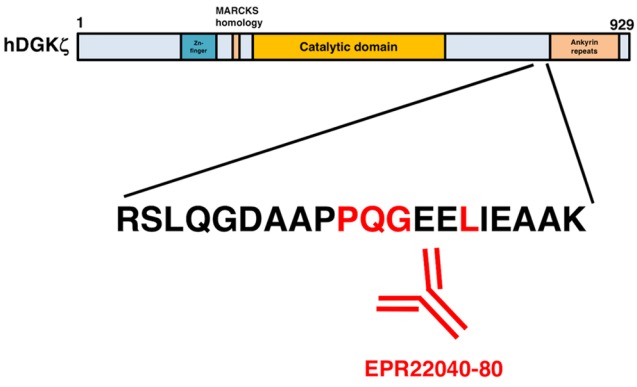

FIG. 2. Schematic illustration of EPR22040-80 epitope. Red amino acids, strong reaction with EPR22040-80.

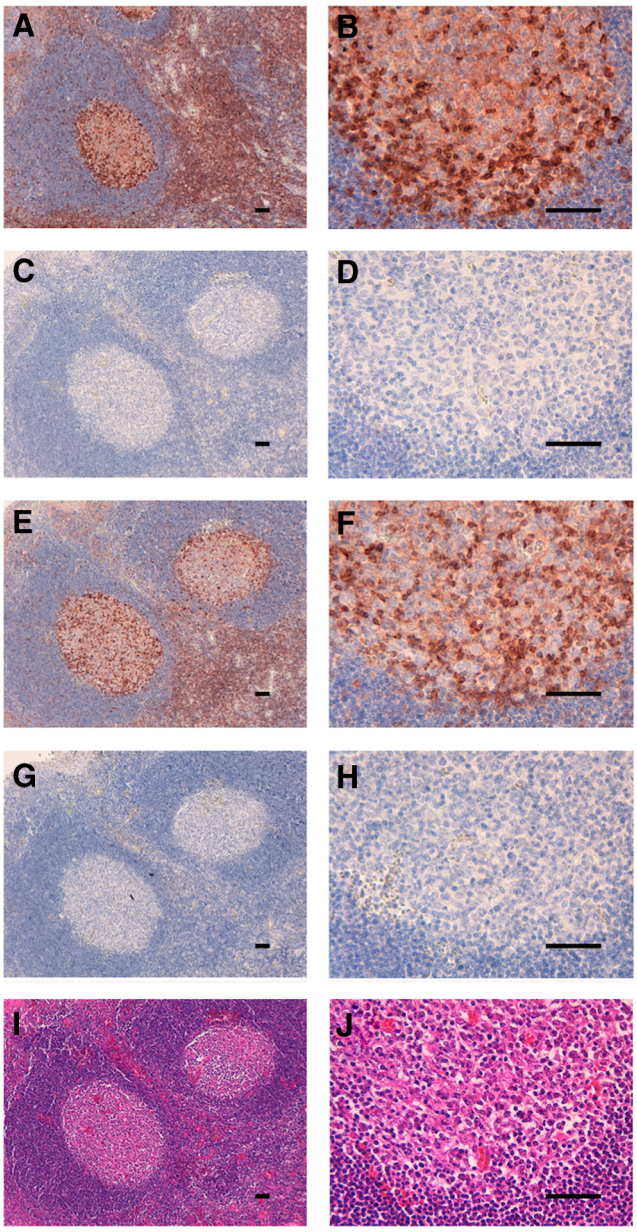

FIG. 3. Inhibition assay. Tissue sections were incubated with EPR22040-80 **(A, B)**, EPR22040-80+R781A **(C, D)**, EPR22040-80+L795A **(E, F)**, or blocking buffer **(G, H)** for 1 hour at room temperature and treated using the Envision+ Kit for rabbit (Agilent Technologies, Inc.) for 30 minutes. Scale bar = 100 μm. **(I, J)** Hematoxylin and eosin staining.

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Author Disclosure Statement

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Address correspondence to:
Yukinari Kato
New Industry Creation Hatchery Center
Tohoku University
2-1, Seiryo-machi
Aoba-ku
Sendai 980-8575
Miyagi
Japan

E-mail: yukinarikato@med.tohoku.ac.jp

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