

Communication

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[Hiroyuki Suzuki](#)^{*}, Yuki Okada, [Tomohiro Tanaka](#), Mika K Kaneko, [Yukinari Kato](#)^{*}

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Communication

Epitope Mapping of an Anti-Mouse CD39 Monoclonal Antibody Using PA Scanning and RIEDL Scanning

Yuki Okada, Hiroyuki Suzuki *, Tomohiro Tanaka, Mika K. Kaneko and Yukinari Kato *

Department of Antibody Drug Development, Tohoku University Graduate School of Medicine,
2-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Miyagi, Japan

* Correspondence: hiroyuki.suzuki.b4@tohoku.ac.jp (H.S.); yukinari.kato.e6@tohoku.ac.jp (Y.K.);
Tel.: +81-22-717-8207 (H.S. & Y.K.)

Abstract: A cell-surface ectonucleotidase CD39 mediates the conversion of extracellular ATP into immunosuppressive adenosine with another ectonucleotidase CD73. The elevated adenosine in the tumor microenvironment (TME) attenuates antitumor immunity, which promotes tumor cell immunologic escape and progression. Anti-CD39 monoclonal antibodies (mAbs), which suppress the enzymatic activity, can be applied to antitumor therapy. Therefore, an understanding of the relationship between the inhibitory activity and epitope of mAbs is important. We previously established an anti-mouse CD39 (mCD39) mAb, C₃₉Mab-1 using the Cell-Based Immunization and Screening (CBIS) method. In this study, we determined the critical epitope of C₃₉Mab-1 using flow cytometry. We performed the PA tag (12 amino acids)-substituted analysis (named PA scanning) and RIEDL tag (5 amino acids)-substituted analysis (named RIEDL scanning) to determine the critical epitope of C₃₉Mab-1 using flow cytometry. By the combination of PA scanning and RIEDL scanning, we identified the conformational epitope, spanning three segments of 275th to 279th, 282nd to 291st, and 306th to 323rd amino acids of mCD39. These analyses would contribute to the identification of the conformational epitope of membrane proteins.

Keywords: mouse CD39; monoclonal antibody; epitope; PA scanning; RIEDL scanning

1. Introduction

In the tumor microenvironment (TME), high concentrations of extracellular ATP (100 to 500 μ M) exist compared to nanomolar order in normal tissues due to the passive release of cell death and active secretion by tumor cells and other subsets.[1–3] The extracellular adenosine, produced by the hydrolysis of extracellular ATP, is involved in immunosuppressive TME [4] and suppresses antitumor immune responses and enhances the immunologic escape of tumor cells [5]. Therefore, the extent of ATP release and its degradation to adenosine should be controlled to restrict the immunosuppressive TME and to facilitate the antitumor immunity during cancer immunotherapy [6,7].

CD39 (ectonucleoside triphosphate diphosphohydrolase 1; encoded by *ENTPD1*) protein has 510 amino acids (aa) and harbors seven potential N-linked glycosylation sites and eleven cysteine residues [8]. Two transmembrane domains exist in the CD39 protein. In the extracellular domain of CD39, five highly conserved segments mediate the enzymatic activity to catalyze the hydrolysis of extracellular ATP and ADP to AMP. Then, CD73 (5'-nucleotidase; encoded by *NT5E*) dephosphorylates AMP into adenosine [9].

Since CD39 mediates the dephosphorylation of extracellular ATP to immunosuppressive adenosine, anti-CD39 monoclonal antibodies (mAbs) have been generated to modulate the adenosine metabolism [6]. A preclinical study showed that B66, an anti-mouse CD39 (mCD39) mAb, can inhibit mCD39 enzymatic activity *in vitro* and exerted the antitumor effect by the mono- or combination therapy with the PD-1 blockade [10]. The anti-human CD39 mAbs, such as TTX-030, IPH5201, and SRF-617, were designed to inhibit the CD39 enzymatic activity [7,10]. These mAbs have been

evaluated in clinical trials for solid tumors in combination with chemotherapeutic agents or immune checkpoint inhibitors [8]. However, the relationship between the inhibitory activity and the epitope has not been clarified.

We previously established a novel anti-mCD39 mAb (C₃₉Mab-1) by the Cell-Based Immunization and Screening (CBIS) method [11–36], and evaluated its applications, including flow cytometry and western blot analyses [37]. In this study, we performed epitope mapping of C₃₉Mab-1 using flow cytometry-mediated novel strategies, named PA scanning and RIEDL scanning.

2. Materials and Methods

2.1. Antibodies

C₃₉Mab-1 (an anti-mCD39 mAb) [37], LpMab-7 (an anti-RIEDL [R*] tag mAb) [38–46], and NZ-1 (an anti-PA tag mAb) [47–53] were described previously. An anti-mCD39 mAb (clone 5F2, mouse IgG₁, kappa) was purchased from BioLegend (San Diego, CA). Alexa Fluor 488-conjugated anti-mouse IgG and anti-rat IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

2.2. Plasmid construction and transfection

mCD39 cDNA was cloned into a pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). For PA scanning, the substitution of PA tag (GVAMPGAEDDVV) in mCD39 was performed with oligonucleotides containing PA tag sequence at the desired position. For example, for the substitution of the PA tag from K288 to P299 of mCD39, we constructed E287-GVAMPGAEDDVV-C300_(288-PA-299) in mCD39. For RIEDL scanning, the substitution of the R* tag in mCD39 was performed with oligonucleotides containing the R* tag sequence at the desired position. For example, for the substitution of the R* tag from E287 to N291 of mCD39, we constructed Y286-RIEDL-V292_(287-R*-291) in mCD39.

Alanine scanning in the mCD39 sequence was performed with oligonucleotides containing the alanine sequence at the desired position. The PCR fragments bearing the desired mutations were inserted into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation) using an In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan).

The mCD39 mutant plasmids were transiently transfected into Chinese hamster ovary (CHO)-K1 cells (the American Type Culture Collection, Manassas, VA) using the Neon Transfection System (Thermo Fisher Scientific Inc., Waltham, MA).

2.3. Flow cytometry

CHO-K1 cells and transfectants were harvested after a brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.) and washed with 0.1% bovine serum albumin in phosphate-buffered saline. C₃₉Mab-1 (1 µg/mL), LpMab-7 (10 µg/mL), or NZ-1 (1 µg/mL) were incubated for 30 min at 4°C. The cells were further treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000 for LpMab-7 and 5F2) or anti-rat IgG (1:2000 for C₃₉Mab-1 and NZ-1). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

3. Results

3.1. Epitope mapping of C₃₉Mab-1 using flow cytometry with PA tag-substituted mCD39

We previously established an anti-mCD39 mAb (C₃₉Mab-1) by the CBIS method.^[37] To determine the C₃₉Mab-1 epitope, we first examined the reactivity to the peptides that cover the extracellular domain of mCD39. However, C₃₉Mab-1 did not react with the peptides (data not shown), suggesting that C₃₉Mab-1 recognizes a conformational and/or modified epitope.

To identify the binding epitope of C₃₉Mab-1, we generated PA tag (GVAMPGAEDDVV)-substituted mCD39 mutants as shown in Figure 1. We analyzed the reactivity of C₃₉Mab-1 against the PA tag-substituted mCD39 (named PA scanning). As shown in Figure 2A, the reactivity of C₃₉Mab-1

almost completely disappeared in 255-PA-266, 267-PA-278, 269-PA-280, 282-PA-293, 288-PA-299, 301-PA-312, 312-PA-323, 325-PA-336, and 415-PA-426 mutants of mCD39. In contrast, the reactivity of C₃₉Mab-1 was observed in the PA tag-substituted mutants from 38th to 253rd, 343rd to 413rd, and 427th to 462nd aa. The cell surface expression of each mutant was confirmed by an anti-PA tag mAb, NZ-1 (Figure 2B). The reactivity was summarized in Figure 1. These results indicated that the epitope of C₃₉Mab-1 contains from 255th to 336th and 415th to 426th aa of mCD39.

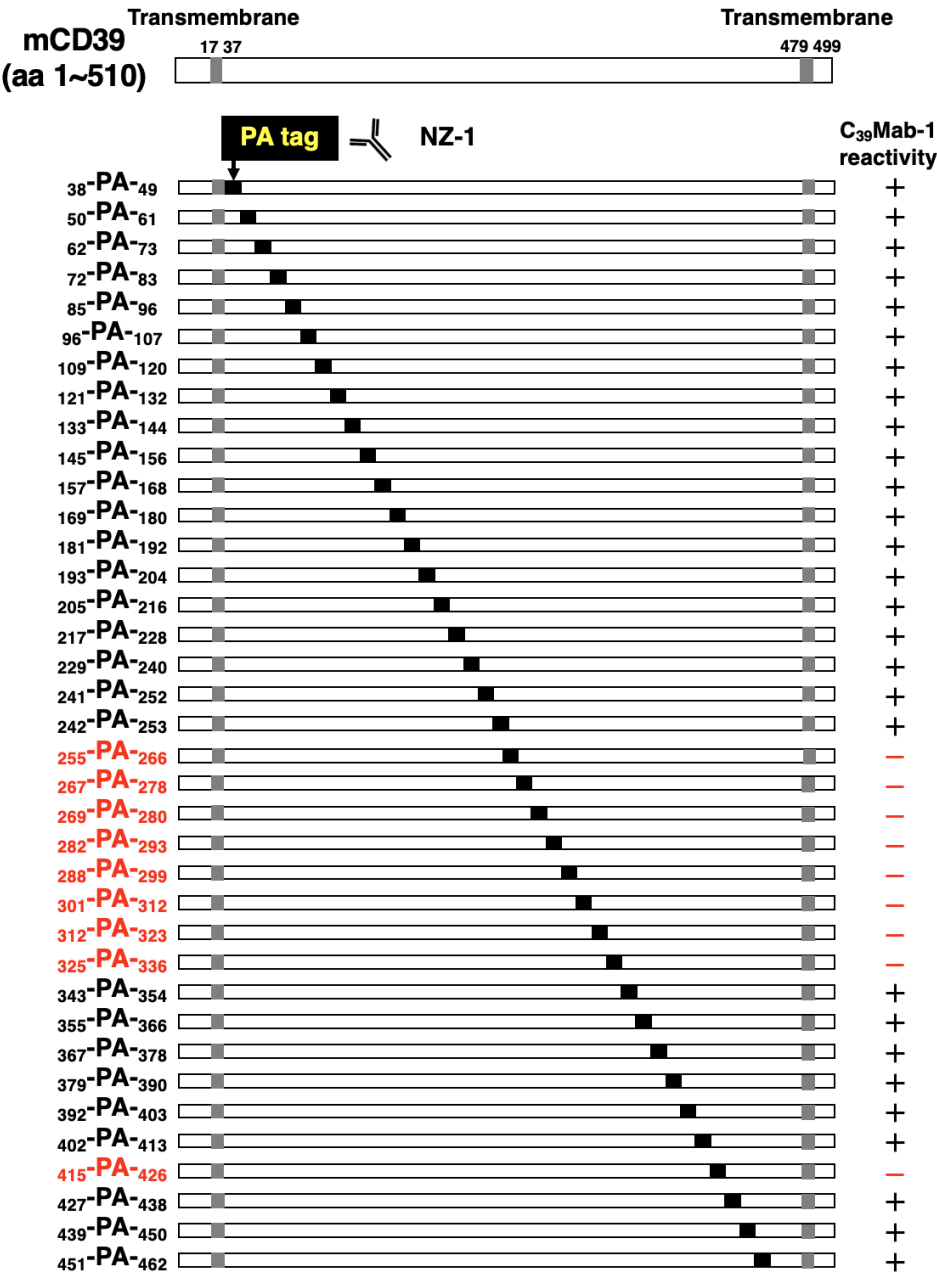


Figure 1. The PA tag-substituted mutants of mCD39. The reactivities of C₃₉Mab-1 are indicated: +, reactive; -, non-reactive.

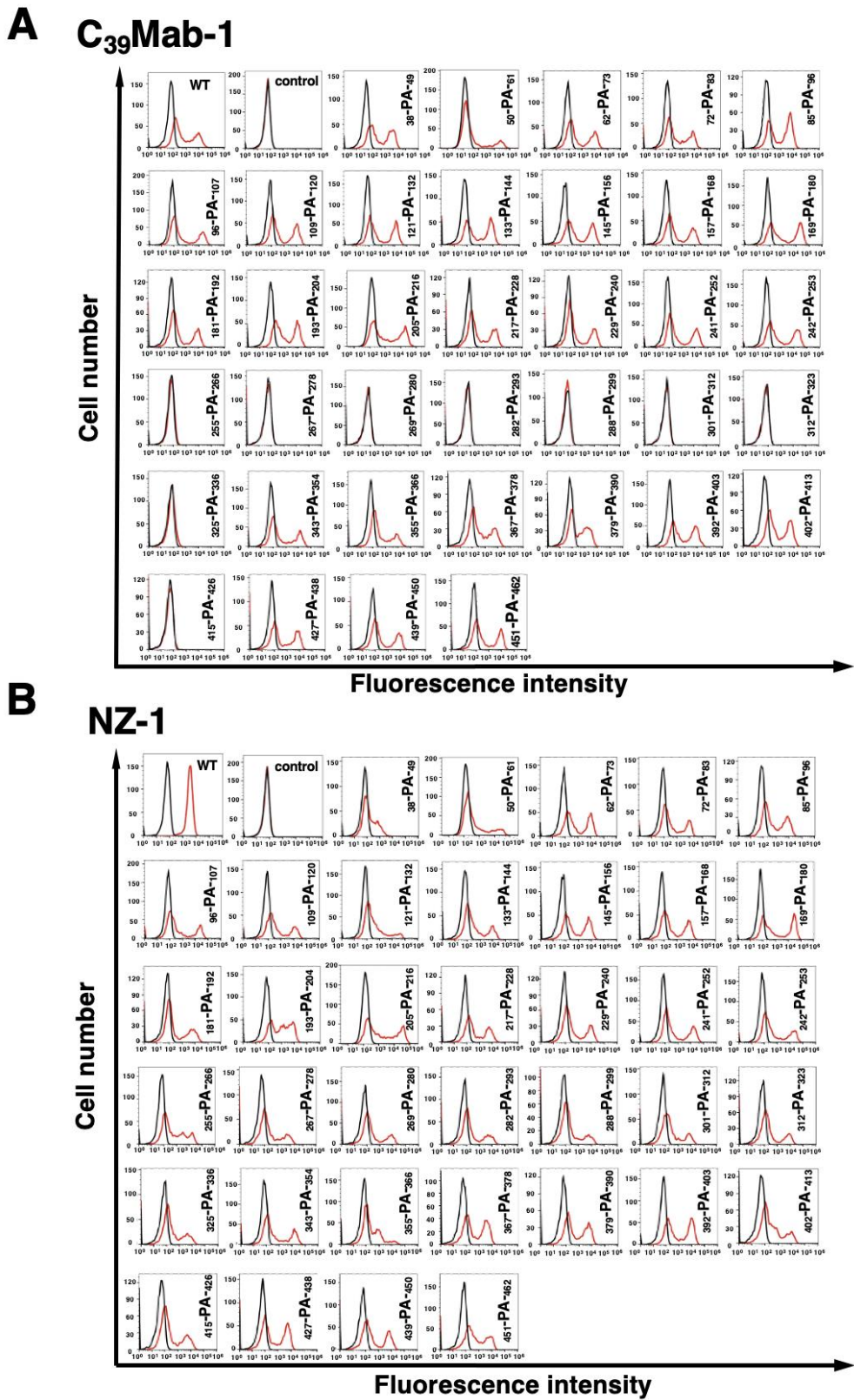


Figure 2. Epitope determination of C₃₉Mab-1 using PA tag-substituted mutants of mCD39. The PA tag-substituted mutants of mCD39 were transiently expressed in CHO-K1 cells. The mutants-expressed CHO-K1 cells were incubated with 1 µg/mL of C₃₉Mab-1 (**A**, red line), 1 µg/mL of NZ-1 (**B**, red line), or control blocking buffer (black line), followed by secondary antibodies treatment. The data were analyzed using the SA3800 Cell Analyzer.

3.2. Epitope mapping of C₃₉Mab-1 using flow cytometry with RIEDL tag-substituted mCD39

As shown in Figure 2A, C₃₉Mab-1 did not react with the continuous PA tag-substituted region from 255th to 336th aa of mCD39. We generated the R* tag-substituted mCD39 as shown in Figure 3 to narrow down the C₃₉Mab-1 epitope. We analyzed the reactivity of C₃₉Mab-1 against the R* tag-substituted mCD39 (named RIEDL scanning). As shown in Figure 4A, the reactivity of C₃₉Mab-1 almost completely disappeared in 275-R*-279, 282-R*-286, 287-R*-291, 306-R*-310, 311-R*-315, 316-R*-320, and 319-R*-323 mutants of mCD39. In contrast, the reactivity of C₃₉Mab-1 was observed in R* tag-substituted mutants from 255th to 274th, 276th to 280th, 292nd to 305th, and 325th to 336th aa. The cell surface expression of each mutant was confirmed by an anti-R* tag mAb, LpMab-7 (Figure 4B). The reactivity was summarized in Figure 3. These results narrowed down the epitope of C₃₉Mab-1 from 275th to 279th, 282nd to 291st, and 306th to 323rd aa of mCD39.

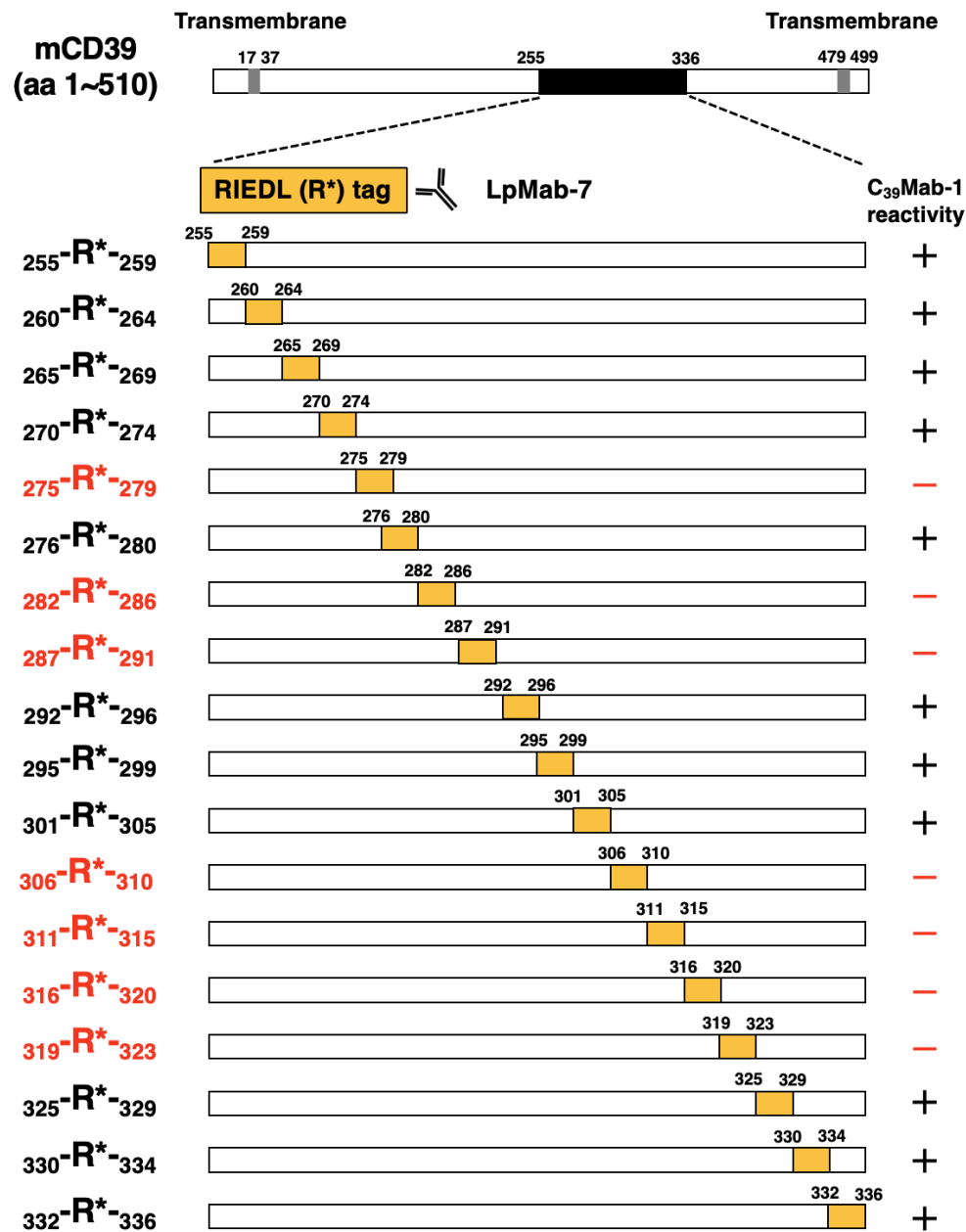


Figure 3. The RIEDL tag-substituted mutants of mCD39. The reactivities of C₃₉Mab-1 are indicated: +, reactive; -, non-reactive.

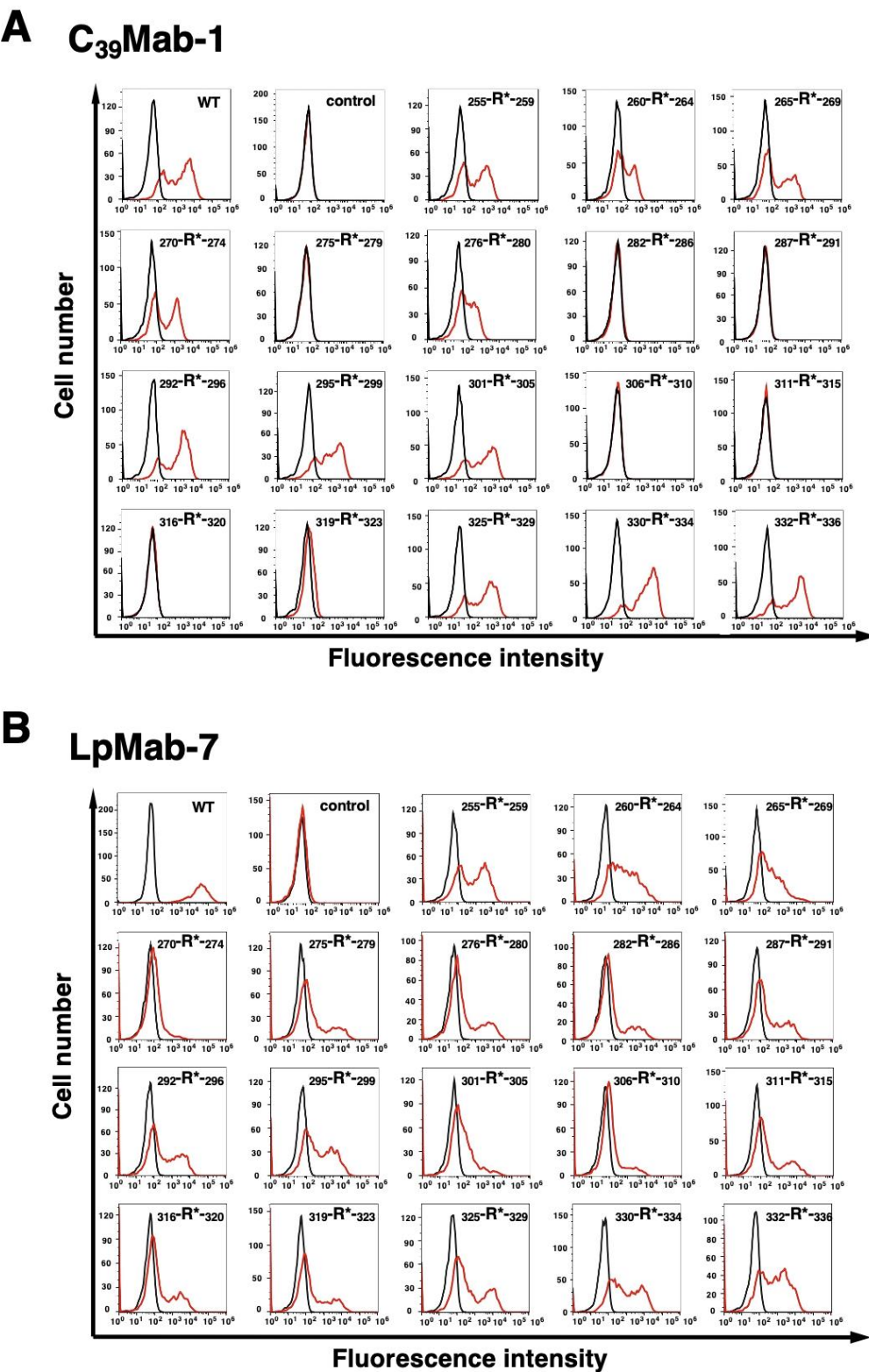


Figure 4. Epitope determination of C₃₉Mab-1 using RIEDL tag-substituted mutants of mCD39. The RIEDL (R*) tag-substituted mutants of mCD39 were transiently expressed in CHO-K1 cells. The mutants-expressed CHO-K1 cells were incubated with 1 µg/mL of C₃₉Mab-1 (**A**, red line), 10 µg/mL of LpMab-7 (**B**, red line), or control blocking buffer (black line), followed by secondary antibodies treatment. The data were analyzed using the SA3800 Cell Analyzer.

3.3. Epitope mapping of C₃₉Mab-1 using flow cytometry with 1×alanine- or 2×alanine-substituted mCD39

The 1×alanine- or 2×alanine-substituted mutant analyses are important strategies to determine the center of the epitope [54–56]. We next generated 33 alanine-substituted mCD39 in 275th to 323rd aa of mCD39 and investigated the reactivity of C₃₉Mab-1 against CHO-K1 cells, which overexpressed the mCD39 mutants transiently. As a result, C₃₉Mab-1 reacted with all alanine-substituted mutants and wild-type (WT) (supplemental Figure 1).

We also examined the reactivity of C₃₉Mab-1 against 2×alanine-substituted mCD39; however, C₃₉Mab-1 reacted with all mutants (supplemental Figure 2). Therefore, we could not determine the epitope of C₃₉Mab-1 using 1×alanine or 2×alanine scanning methods.

4. Discussion

In this study, we performed the flow cytometry-based epitope mapping of C₃₉Mab-1 using the PA scanning (Figures 1 and 2) and the RIEDL scanning (Figures 3 and 4). We found that the three segments, 275th to 279th, 282nd to 291st, and 306th to 323rd aa of mCD39 are important for the recognition by C₃₉Mab-1.

Zebisch *et al.* demonstrated the crystal structure of rat CD39 between the two lobes of the catalytic domain [57]. Figure 5A shows the structure of rat CD39 (PDB ID: 3ZX3). Apyrase-conserved regions (ACR1-5) form the active-site cleft [58], which is distant from the three segments identified as C₃₉Mab-1 epitope. As shown in Figure 5B, the corresponding rat CD39 sequence to the three segments forms a β -sheet structure. In mouse sequence motifs, 276-VLKD-279, 287-EKVVN-291, and 312-QFRIQG-317 could contribute to the formation of the β -sheet (Figure 5C). The substitution of the R* tag on not only the above three motifs but also surrounding sequence may disrupt the β -sheet structure, which results in the impaired recognition by C₃₉Mab-1. Although we could not determine the critical aa of the epitope by 1×alanine or 2×alanine scanning methods (supplemental Figures 1 and 2), it would be interesting to introduce mutations between some β -sheet segments and examine the reactivity of C₃₉Mab-1.

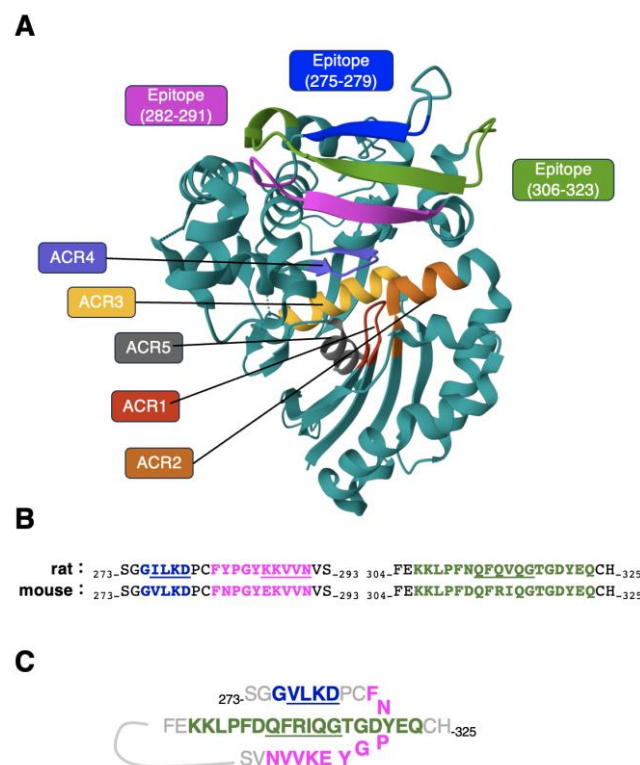


Figure 5. Structure of CD39 and putative epitope of C₃₉Mab-1. (A) Rat CD39 structure (PDB ID: 3ZX3). The corresponding sequence to the three segments of C₃₉Mab-1 epitope segments is highlighted. Apyrase-conserved regions (ACR1-5) are also indicated. (B) The alignment of rat and

mouse CD39 sequence around C₃₉Mab-1 epitope segments. The underlined sequences form a β -sheet as shown in (A). (C) Putative β -sheet structure of C₃₉Mab-1 epitope segments.

Therapeutic anti-CD39 mAbs, including TTX-030, were designed to inhibit CD39 enzymatic activity through the uncompetitive allosteric mechanism of action [7,10]. The epitope of TTX-030 was determined as E142 to Y159 using the human-mouse CD39 chimeric protein by flow cytometry [59]. The region is distal to the ATP-binding residues (E174 and S218), supporting the allosteric mechanism of action by TTX-030. However, the optimal mAb-binding sites to inhibit the CD39 enzymatic activity have not been identified. Therefore, the detailed relationship between each mAb epitope and the inhibitory activity should be determined. The PA scanning and RIEDL scanning would contribute to the determination of the conformational epitope of not only CD39 but also other membranous antigens.

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