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# Epitope Mapping of PMab-241, a Lymphatic Endothelial Cell-Specific Anti-Bear Podoplanin Monoclonal Antibody

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Anti-bear podoplanin (bPDPN) monoclonal antibodies (mAbs), including PMab-247 and PMab-241, have been previously established. Although PMab-247 has shown positive immunostaining for lymphatic endothelial cells (LECs), type I alveolar cells of the lung, and podocytes of the kidney, PMab-241 stains LECs but does not react with lung type I alveolar cells. PDPN possesses three platelet aggregation-stimulating (PLAG) domains (PLAG1, PLAG2, and PLAG3) and the PLAG-like domain (PLD). The binding epitope of PMab-247 was previously determined to include bPDPN residues Asp76, Arg78, Glu80, and Arg82. Among these, Glu80 and Arg82 are included in PLD of bPDPN. The purpose of this study is to determine the binding epitope of PMab-241 and to clarify the difference between these two anti-bPDPN mAbs. Analysis of bPDPN deletion mutants revealed that the N-terminus of the PMab-241 epitope exists between amino acids (aa) 75 and 80 of bPDPN. In addition, analysis of bPDPN point mutants demonstrated that the critical epitope of PMab-241 includes Thr75, Asp76, and Arg78 of bPDPN. The binding epitopes of PMab-241 and PMab-247 seem to overlap, but this slight difference may be sufficient to provide the specificity of PMab-241 to discriminate LECs from type I alveolar cells of the lung.

**Keywords:** bear podoplanin, monoclonal antibody, epitope, PMab-241

## Introduction

Sensitive and specific monoclonal antibodies (mAbs) against podoplanin (PDPN) of various species, including human,[1] experimental animals,[2] and wild animals,[3] are in high demand to analyze the expression and pathophysiological function of PDPN. PDPN is used for discriminating lymphatic endothelial cells (LECs) from vascular endothelial cells[4] or type I alveolar cells of the lung from type II alveolar cells of the lung.[5] We recently developed anti-bear podoplanin (bPDPN) mAbs, including PMab-247[6] and PMab-241,[7] using the Cell-Based Immunization and Screening (CBIS) method.[8–10] Similar to other typical anti-PDPN mAbs, PMab-247 demonstrated positive immunoreaction[11] for LECs,[4] type I alveolar cells of the lung,[5] and podocytes of the kidney.[12] By contrast, PMab-241 stained LECs but did not react with type I alveolar cells of the lung,[7] suggesting that the binding epitope of PMab-247 is different from that of PMab-241.

To identify the binding epitope of PMab-247, we previously produced deletion mutants and point mutants of bPDPN and clearly showed that the PMab-247 epitope is Asp76, Arg78, Glu80, and Arg82 of bPDPN.[13] PDPN is also known as Aggrus,[14] a platelet aggregation-inducing factor that comprises three platelet aggregation-stimulating (PLAG) domains (PLAG1, PLAG2, and PLAG3)[15] at the N-terminus and PLAG-like domains (PLDs)[3,13,16–18] in the middle of the PDPN protein. Among these, Glu80 and Arg82 are included in PLD of bPDPN. However, the PMab-241 epitope has not been clarified.

The aim of this study was to determine the binding epitope of PMab-241 and to uncover the difference between the two anti-bPDPN mAbs.

## Materials and Methods

### *Cell lines*

Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). The bPDPN mutation plasmids containing bovine Aggrus/podoplanin (BAP) tag[6] were transfected into CHO-K1 cells using Lipofectamine LTX (Thermo Fisher Scientific, Inc., Waltham, MA). The BAP tag comprises 16 amino acids (aa) (EKTTLGVEDYTTTPAA), and is detected by PMab-44 mAb.[19] Cells transiently transfected with deletion mutants or point mutants were cultured in Roswell Park Memorial

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Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 $\mu$g/mL of streptomycin, and 25 $\mu$g/mL of amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

### *Production of bPDPN mutants*

Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding bPDPN (Accession No. XM_008694703.1) was subcloned into the pCAG vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and a BAP tag was added at the N-terminus. Deletion mutants of the bPDPN sequence were produced using a HotStar HiFidelity Polymerase Kit (Qiagen, Inc., Hilden, Germany) with oligonucleotides. Substitutions of amino acids to alanine in the bPDPN sequence were conducted by QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, Inc., Santa Clara, CA). Polymerase chain reaction fragments bearing the desired mutations were inserted into the pCAG vector using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan).

### *Flow cytometry*

Transiently transfected CHO-K1 cells were detached by 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.) and were collected using 10% FBS in RPMI 1640 medium. After washing with 0.1% bovine serum albumin/phosphate buffered saline, the cells were incubated with an anti-bPDPN antibody (PMab-241; 1 $\mu$g/mL) or an anti-BAP tag antibody (PMab-44; 1 $\mu$g/mL) for 30 minutes at 4°C. Alexa Fluor 488-conjugated anti-mouse IgG (1:2000; Cell Signaling Technology, Inc., Danvers, MA) for detection of PMab-241 and PMab-44 was added to each cell and was incubated for 30 minutes at 4°C. Fluorescence data were collected and analyzed using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

## Results

We generated seven deletion mutants of bPDPN in CHO-K1 cells, namely dN30 (corresponding to 30–160 aa), dN40 (corresponding to 40–160 aa), dN50 (corresponding to 50–160 aa), dN60 (corresponding to 60–160 aa), dN70 (corresponding to 70–160 aa), dN75 (corresponding to 75–160 aa), and dN80 (corresponding to 80–160 aa) or wild type (WT) bPDPN (corresponding to 27–160 aa). All deletion mutants and WT of bPDPN containing the N-terminal BAP tag were detected by PMab-44 (an anti-BAP tag mAb), indicating that the expression level of each construct is very high (Fig. 1A). Although PMab-241 detected dN30, dN40, dN50, dN60, dN70, and dN75, it did not react with dN80 (Fig. 1B),

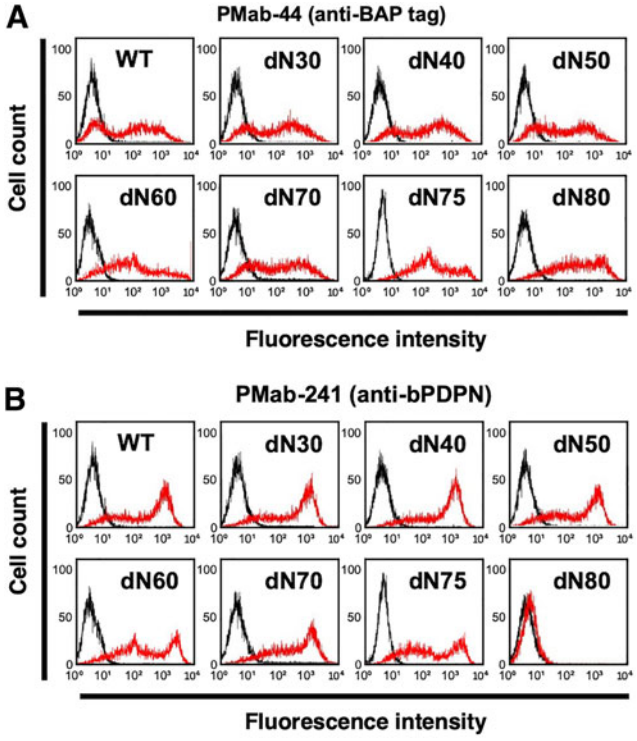


**FIG. 1.** Epitope mapping of PMab-241 using deletion mutants of bPDPN. Deletion mutants of bPDPN were analyzed using flow cytometry. Mutants were incubated with PMab-44 (anti-BAP tag; red line, **A**), PMab-241 (anti-bPDPN mAb; red line, **B**), or buffer control (black line, **A, B**) for 30 minutes at 4°C, followed by secondary antibodies. BAP, bovine Aggrus/podoplanin; bPDPN, bear podoplanin; mAbs, monoclonal antibodies.

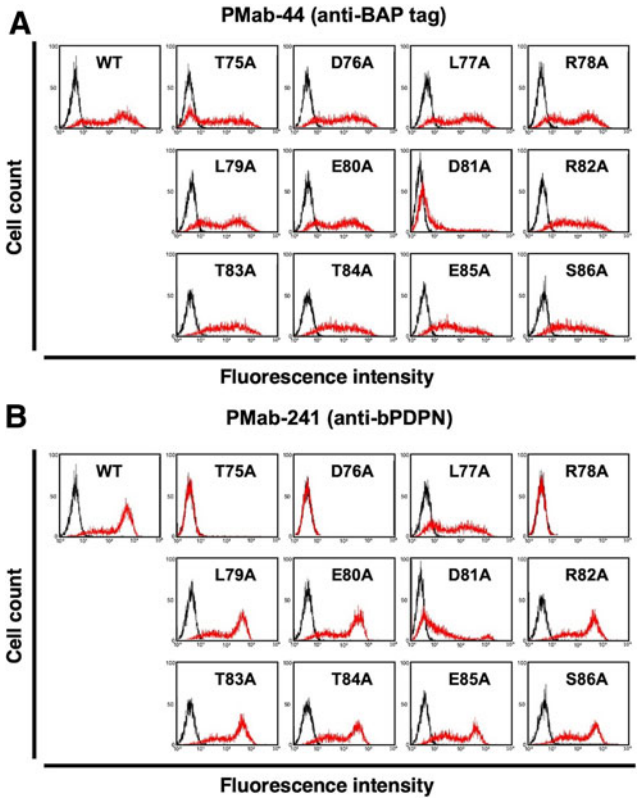


**FIG. 2.** Epitope mapping of PMab-241 using point mutants of bPDPN. Transient point mutants expressing T75A, D76A, L77A, R78A, L79A, E80A, D81A, R82A, T83A, T84A, E85A, or S86A of bPDPN were incubated with PMab-44 (red line, **A**), PMab-241 (red line, **B**), or buffer control (black line, **A, B**) for 30 minutes at 4°C, followed by secondary antibodies.

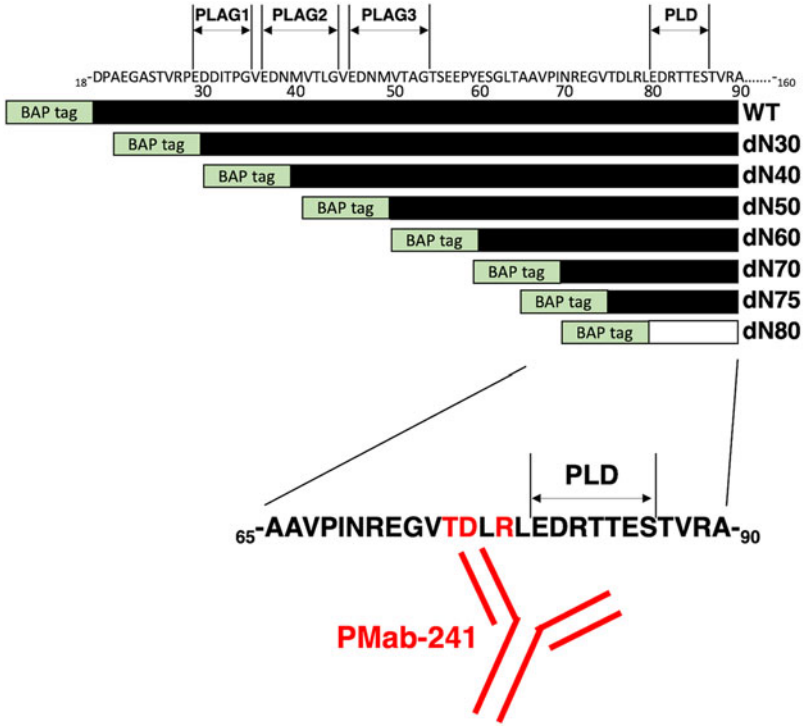


**FIG. 3.** Schematic illustration of the epitope recognized by PMab-241. Illustration of WT bPDPN and its seven deletion mutants: dN30, dN40, dN50, dN60, dN70, dN75, and dN80. Black bars: positive reactions of PMab-241. White bar: negative reactions of PMab-241. Red amino acids indicate a critical epitope of PMab-241. PLAG, platelet aggregation-stimulating domain; PLD, PLAG-like domain; WT, wild type.

suggesting that the N-terminus of the PMab-241 epitope exists between bPDPN aa 75 and 80.

Next, to determine the binding epitope of PMab-241, we produced the following series of point mutants of bPDPN: T75A, D76A, L77A, R78A, L79A, E80A, D81A, R82A, T83A, T84A, E85A, and S86A. PMab-44 reacted with all point mutants (Fig. 2A). By contrast, PMab-241 did not react with T75A, D76A, and R78A (Fig. 2B). These results indicated that Thr75, Asp76, and Arg78 of bPDPN are essential for PMab-241 binding to bPDPN (Fig. 3).

## Discussion

Highly sensitive and specific mAbs against not only human but also experimental animals and wild animals are necessary to analyze the expression and pathophysiological function of PDPN. Thus, we have developed mAbs against human,[20] mouse,[21] rat,[22] rabbit,[23] dog,[24] cat,[25] bovine,[26] pig,[27,28] Tasmanian devil,[29] alpaca,[30] tiger,[31] whale,[32] goat,[33,34] horse,[35,36] and bear[7,11] PDPNs using the CBIS method.[8–10] Among these antibodies, we have shown that anti-bPDPN mAb PMab-247 is very useful for immunohistochemical analyses for detecting LECs and type I alveolar cells of the bear lung.[6] We sometimes need to discriminate LECs from vascular endothelial cells in each organ; PDPN is used for the specific marker for LECs. However, it is very difficult to detect LECs in lung because PDPN is also highly expressed in type I alveolar cells. PMab-247 reacts with both LECs and type I alveolar cells[11]; therefore, a specific mAb for LECs, not reacting with type I alveolar cells is advantageous in pathological diagnosis.

After screening of many anti-bPDPN mAbs, we finally established a LEC-specific anti-bPDPN mAb, PMab-241.[7] In this study, we investigated the binding epitope of PMab-241 and compared it with that of PMab-247. We previously demonstrated that the PMab-247 epitope is Asp76, Arg78, Glu80, and Arg82 of bPDPN.[13] Because Glu80 and Arg82 are included in the PLD of bPDPN, PMab-247 may inhibit platelet aggregation by bPDPN. We herein clarified that Thr75, Asp76, and Arg78 of bPDPN are critical epitope components for PMab-241, and these residues are not located in PLAG and/or PLD. We likewise hope to establish in future studies LEC-specific anti-PDPN mAbs against other species for the pathophysiological analysis of LECs in lungs or other organs.

## Author Disclosure Statement

No competing financial interests exist.

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