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Epitope Analysis of an Anti-Whale Podoplanin Monoclonal Antibody, PMab-237, Using Flow Cytometry

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Podoplanin (PDPN) is a small mucin-type transmembrane glycoprotein, which was first discovered in podocytes of the kidney. PDPN is a specific lymphatic endothelial marker and is also known as T1alpha, a marker of lung type I alveolar cells, or Aggrus, a platelet aggregation-inducing factor. PDPN possesses three platelet aggregation-stimulating (PLAG) domains and PLAG-like domains (PLDs), which bind to C-type lectin-like receptor-2. Previously, we developed a novel anti-whale PDPN (wPDPN) monoclonal antibody (mAb) PMab-237 using the Cell-Based Immunization and Screening (CBIS) method and the RIEDL tag of Arg-Ile-Glu-Asp-Leu sequence. PMab-237 detected wPDPN by flow cytometry, western blot, and immunohistochemical analyses. However, the specific binding epitope of PMab-237 for wPDPN remains unknown. In this study, deletion mutants and point mutants of wPDPN with N-terminal RIEDL tag were produced to analyze the PMab-237 epitope using flow cytometry. The analysis of deletion mutants showed that the N-terminus of the PMab-237 epitope exists between the 80th amino acid (AA) and the 85th AA of wPDPN. In addition, the analysis of point mutants demonstrated that the critical epitope of PMab-237 includes Leu82 and Thr84 of wPDPN, indicating that the PMab-237 epitope is located in the PLD of wPDPN.

Keywords: whale, podoplanin, monoclonal antibody, epitope, PMab-237

Introduction

Podoplanin (PDPN)/T1alpha/Aggrus/PA2.26 is a type I transmembrane sialoglycoprotein consisting of a heavily glycosylated extracellular domain, a single transmembrane, and a short nine amino acid (AA) cytoplasmic tail.[1–4] PDPN/Aggrus possesses the EDxxVTPG sequence at its N-terminus, which is known to be the platelet aggregation-stimulating (PLAG) domains (PLAG1, PLAG2, and PLAG3).[3,5] In addition, the PLAG-like domain (PLD) of the E(D/E)xx(T/S)xx sequence, also known as PLAG4, has been reported to be present in the middle of PDPN.[6,7] PLAG domains are highly conserved among mammalian PDPNs.[7]

PDPN is expressed in lymphatic endothelial cells and is not expressed in vascular endothelial cells.[8] The interaction between PDPN[3] on lymphatic endothelial cells and C-type lectin-like receptor-2 on platelets was shown to facilitate embryonic blood/lymphatic vessel separation.[9] Because PDPN/T1alpha is expressed in type I alveolar cells but not in type II alveolar cells, it is used as a specific marker of type I alveolar cells.[10] In recent studies, other functions of PDPN were reported. The PDPN-positive cells, with the immune cells after myocardial infarction, positively affect immune cell recruitment.[11] The PDPN-positive stromal cells play a critical role in a network of immunofibroblasts, which can support the earliest phases of tertiary lymphoid structure establishment.[12] The expression of PDPN in chorionic villous stromal cells is in two important placental pathologies: preeclampsia and hydatidiform mole.[13] Moreover, PDPN is upregulated in many cancers and is involved in cancer metastasis and malignant progression.[14–17] Recent reports showed that PDPN is related to a progression in oral epithelial dysplasia and oral squamous cell carcinoma through a co-expression with sex-determining region Y-related Homeo box gene 2.[18,19] Therefore, PDPN possesses many pathophysiological functions in malignant tissues.

Recently, we developed a novel anti-whale PDPN (wPDPN) monoclonal antibody (mAb), PMab-237, using the Cell-Based Immunization and Screening (CBIS) method.[20] The CBIS method was established in our previous study[21] to produce mAbs using cell lines for immunization and screening. PMab-237 specifically detected wPDPN by flow cytometry, western blotting, and immunohistochemical analyses. PMab-237 also strongly stained pulmonary type I alveolar cells, renal podocytes, and lymphatic endothelial cells of the harbor porpoise by the immunohistochemical analysis.[22] However, the binding epitope of PMab-237 for wPDPN remains unknown. This study aimed to identify the epitope of PMab-237

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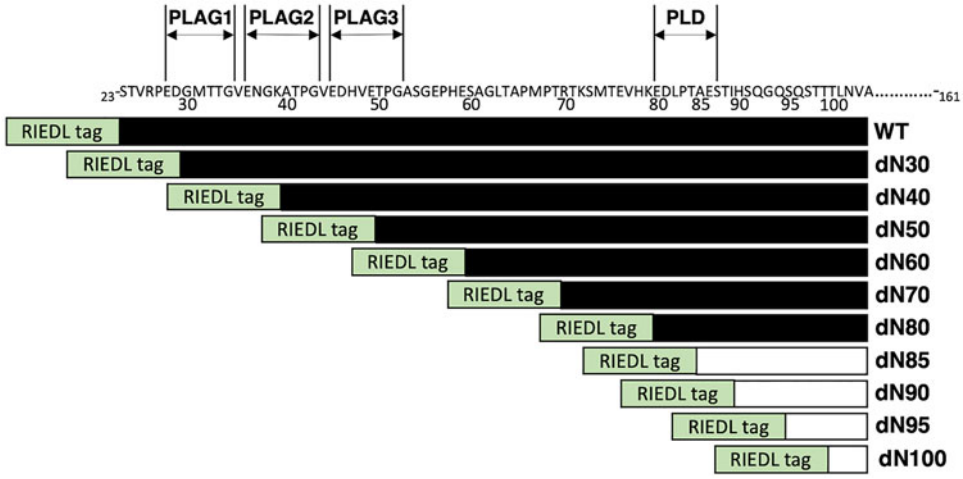

FIG. 1. Schematic illustration of deletion mutants of anti-wPDPN. Illustration of WT and 10 deletion mutants of wPDPN: dN30, dN40, dN50, dN60, dN70, dN80, dN85, dN90, dN95, and dN100. Black bars: the positive reaction of PMab-237. White bars: the negative reaction of PMab-237. PLAG, platelet aggregation-stimulating; PLD, PLAG-like domain; wPDPN, whale podoplanin; WT, wild type.

through flow cytometry using the deletion mutants and point mutants of wPDPN.

Materials and Methods

Production of wPDPN mutants

Synthesized DNA encoding wPDPN was subcloned into the pCAG vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and an N-terminal RIEDL tag was added.[22] The RIEDL tag was derived from the five AA sequences (Arg-Ile-Glu-Asp-Leu) of human PDPN, which was detected by clone LpMab-7.[23] Deletion mutants of the wPDPN sequence were produced using a HotStar HiFidelity Polymerase Kit (Qiagen, Inc., Hilden, Germany) with oligonucleotides. Substitutions of AAs to alanine in the wPDPN sequence were conducted by QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, Inc., Santa Clara, CA). PCR fragments bearing the desired mutations were inserted into the pCAG vector using an In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan).

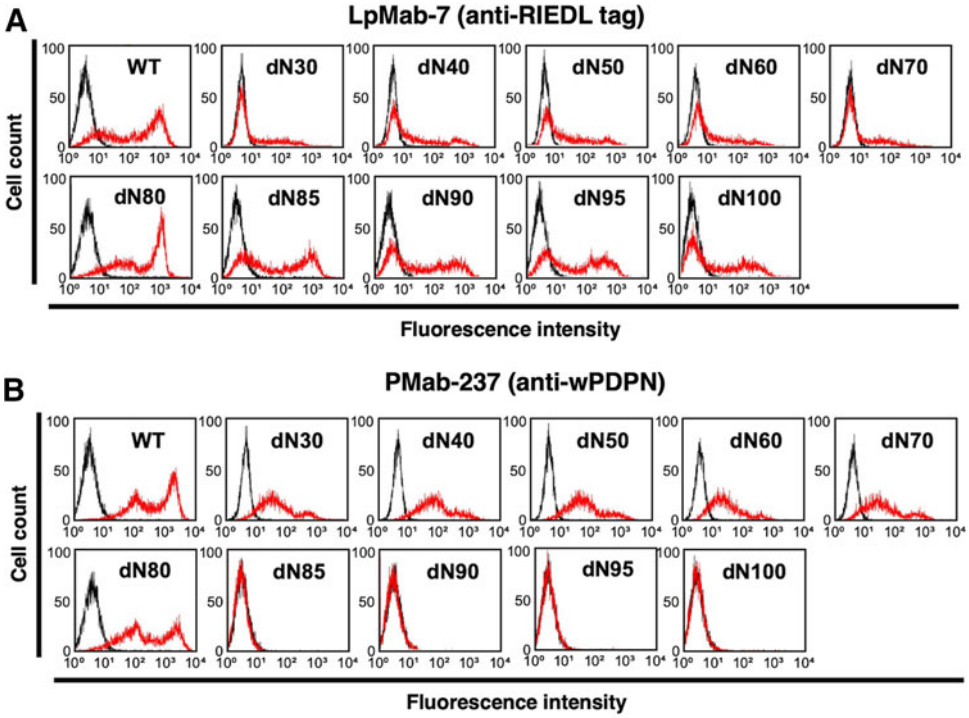

FIG. 2. Epitope mapping of PMab-237 using deletion mutants of wPDPN. Deletion mutants of wPDPN were analyzed using flow cytometry. Deletion mutants were incubated with LpMab-7 (anti-RIEDL tag; red line, **A**), PMab-237 (anti-wPDPN mAb; red line, **B**), or buffer control (black line, **A**, **B**) for 30 minutes at 4°C, followed by secondary antibodies. mAb, monoclonal antibody.

Cell lines and culture condition

Chinese hamster ovary (CHO)-K1 was obtained from the American Type Culture Collection (Manassas, VA). The wPDPN mutation plasmids containing the RIEDL tag were transfected into CHO-K1 cells using Lipofectamine LTX (Thermo Fisher Scientific, Inc., Waltham, MA). Transiently transfected cells with deletion mutants or point mutants were cultured in 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 μg/mL of streptomycin, and 25 μg/mL of amphotericin B (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Flow cytometry

Transiently transfected CHO-K1 cells were detached by 0.25% trypsin/1 mM of ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.), and were collected using 10% FBS in RPMI 1640 medium. After washing with 0.1% bovine serum albumin and phosphate-buffered saline, the cells were incubated with an anti-wPDPN antibody (PMab-237[22]; 1 μg/mL) or an anti-RIEDL tag antibody (LpMab-7[23]; 1 μg/mL) for 30 minutes at 4°C. Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA) for detection of PMab-237 and LpMab-7 were added to each cell and incubated for 30 minutes at 4°C. Fluorescence data were collected and analyzed using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Results

Epitope analysis using deletion mutants of wPDPN

Ten deletion mutants of wPDPN, such as dN30 (corresponding to 30–161 AA), dN40 (corresponding to 40–161 AA), dN50 (50–161 AA), dN60 (60–161 AA), dN70 (70–161 AA), dN80 (80–161 AA), dN85 (85–161 AA), dN90 (90–161 AA), dN95 (95–161 AA), and dN100 (100–161 AA), or wild type (WT) of wPDPN (corresponding to 23–161 AA) were generated using CHO-K1 cells (Fig. 1).

All deletion mutants and WT of wPDPN containing an N-terminal RIEDL tag were detected by LpMab-7 (an anti-RIEDL tag mAb), indicating that the expression level of each

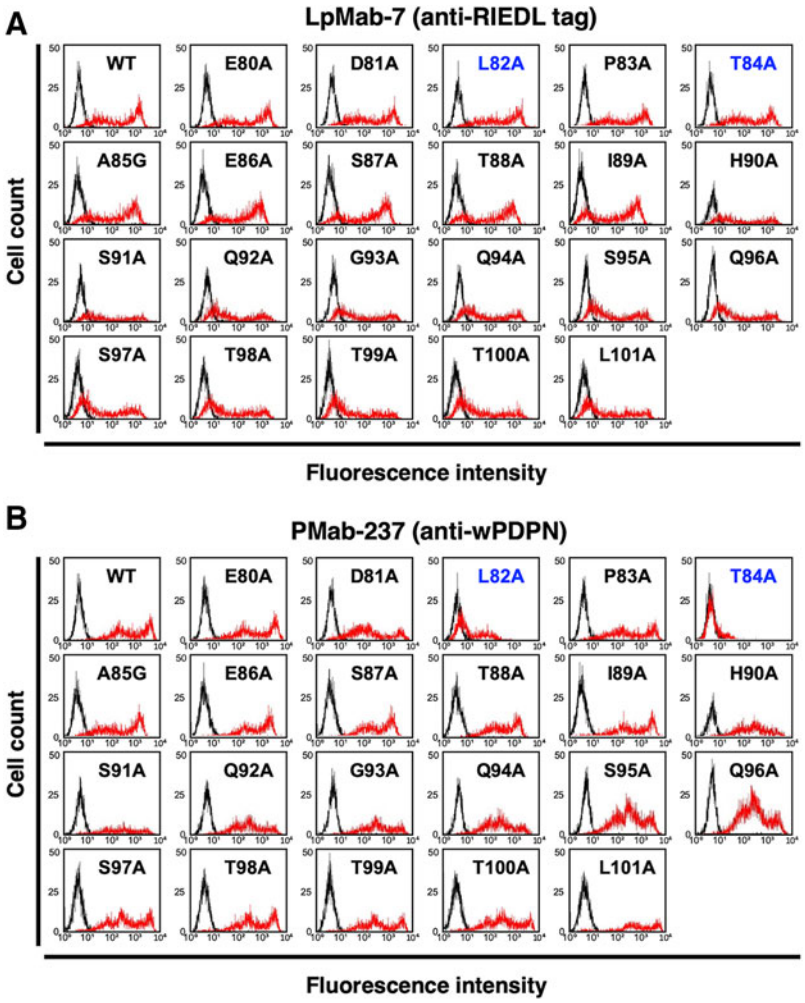

FIG. 3. Epitope mapping of PMab-237 using point mutants of wPDPN. Transient point mutants expressing E80A, D81A, L82A, P83A, T84A, A85G, E86A, S87A, T88A, I89A, H90A, S91A, Q92A, G93A, Q94A, S95A, Q96A, S97A, T98A, T99A, T100A, and L101A of wPDPN were incubated with LpMab-7 (red line, **A**), PMab-237 (red line, **B**), or buffer control (black line, **A**, **B**) for 30 minutes at 4°C, followed by secondary antibodies.

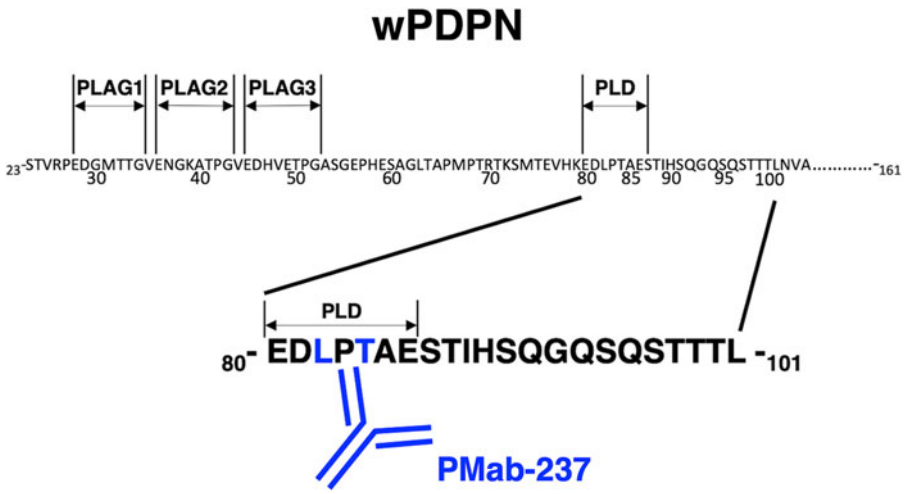

FIG. 4. Schematic illustration of the epitope recognized by PMab-237. The critical epitope of PMab-237 includes Leu82 and Thr84 of wPDPN. Both Leu82 and Thr84 are included in the PLD.

construct was high (Fig. 2A). In contrast, PMab-237 did not react with dN85, dN90, dN95, or dN100 (Fig. 2B), suggesting that the N-terminus of the PMab-237 epitope might exist between the 80th AA and 85th AA of wPDPN.

Epitope analysis using point mutants of wPDPN

To identify the binding epitope of PMab-237, we then produced a series of point mutants of wPDPN using CHO-K1 cells, including E80A, D81A, L82A, P83A, T84A, A85G, E86A, S87A, T88A, I89A, H90A, S91A, Q92A, G93A, Q94A, S95A, Q96A, S97A, T98A, T99A, T100A, and L101A.

LpMab-7 reacted with all point mutants, indicating that all transfectants express wPDPN (Fig. 3A). In contrast, PMab-237 weakly recognized L82A and did not react with T84A, indicating that the L82A and T84A of wPDPN could be included in the critical epitope of PMab-237 (Fig. 3B). Taken together, the epitope of PMab-237 is located in the PLD of wPDPN (Fig. 4).

Discussion

We have demonstrated that the critical epitope of PMab-237 could include Leu82 and Thr84 of wPDPN using the deletion mutants and point mutants of wPDPN in CHO-K1 cells by flow cytometry. In our previous studies, we developed many mAbs against PDPNs of human,[24] mouse,[25] rat,[26] rabbit,[27] dog,[28] cat,[29] bovine,[30] horse,[31] Tasmanian devil,[32] alpaca,[33] bear,[34] tiger,[35] goat,[36] pig,[37,38] and whale.[20] We successfully determined the binding epitope of those mAbs.[22,34,39–48] These epitope mapping results showed that almost all anti-PDPN mAbs react with PLAG domains or PLDs.[7,39–41,43,44,49–51] The critical epitope of PMab-237 was also shown to be located in PLD (Fig. 4), suggesting that PLAG domains and PLD were advantageous to the epitope for several applications such as flow cytometry, western blotting, and immunohistochemical analyses.

Furthermore, using glycan-deficient CHO cell lines, such as Lec1 (*N*-glycan-deficient), Lec2 (sialic acid-deficient), or Lec8 (galactose-deficient),[52] we investigated whether the epitope of PMab-237 could include not only AAs but also glycans. A flow cytometric analysis demonstrated that PMab-237 reacts not only with CHO-K1/wPDPN but also with Lec1/wPDPN, Lec2/wPDPN, and Lec8/wPDPN (data not shown), indicating that glycans of wPDPN might not be included in the PMab-237 epitope. This analysis further suggests that no glycan is attached to Thr84 in the PLD of wPDPN (Fig. 4).

In conclusion, by using deletion mutants and point mutants of wPDPN in CHO-K1 cells, we have demonstrated that the critical epitope of PMab-237 may include Leu82 and Thr84 of wPDPN. PMab-237 could be a useful tool in elucidating the pathophysiological function of wPDPN.

Author Disclosure Statement

No competing financial interests exist.

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