MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 41, Number 4, 2022

DOI: 10.1089/mab.2022.0017

Epitope Mapping of an Anti-elephant Podoplanin Monoclonal Antibody (PMab-295) Using Enzyme-Linked Immunosorbent Assay

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Podoplanin (PDPN) is a marker of lung type I alveolar cells, kidney podocytes, and lymphatic endothelial cells. The overexpression of PDPN contributes to the malignant progression of tumors. Therefore, the development of anti-PDPN monoclonal antibodies (mAbs) to animals is essential to evaluate the pathogenesis and cellular functions. Using peptide immunization, we previously developed an anti-elephant PDPN (elePDPN) mAb, PMab-295, which is useful for flow cytometry, Western blotting, and immunohistochemistry. In this study, we determined the critical epitope of PMab-295 by enzyme-linked immunosorbent assay (ELISA). We performed ELISA with the alanine-substituted peptides of elePDPN extracellular domain (amino acids 38–51), and found that PMab-295 did not recognize the alanine-substituted peptides of M41A, P44A, and E47A. Furthermore, these peptides could not inhibit the recognition of PMab-295 to elePDPN-expressing cells by flow cytometry and immunohistochemistry. The results indicate that the binding epitope of PMab-295 includes Met41, Pro44, and Glu47 of elePDPN.

Keywords: elephant podoplanin, epitope mapping, monoclonal antibody, enzyme-linked immunosorbent assay

Introduction

Podoplanin (PDPN) is a type I transmembrane mucin-like glycoprotein that plays critical roles in normal lung,[1] kidney,[2] and lymphatic vessels.[3] In humans, PDPN has been reported to be overexpressed in cancers, and plays crucial roles in the malignant progression.[4] PDPN is also upregulated in tumor microenvironment, including cancer-associated fibroblasts (CAFs)[5] and lymphocytes.[6] CAFs play a critical role in the formation of immunosuppressive tumor microenvironment[7,8] and remodeling of the extracellular matrix. Therefore, anti-PDPN monoclonal antibodies (mAbs) are useful to identify the specific cells in tissue staining, and investigate the cellular functions in PDPN-expressing cancer cells.

The N-terminal extracellular domain of PDPN has a repeat sequence of platelet aggregation-stimulating (PLAG)1 to PLAG3 domains,[9] which interacts with a platelet receptor, C-type lectin-like receptor 2, and promotes platelet aggregation.[10,11] Furthermore, several PLAG-like domains (PLD) with similar sequences were identified at the central region of PDPN.[4,12] In the cytoplasmic tail, PDPN interacts with ezrin, radixin, and moesin family proteins and regulates cell proliferation, migration, invasion, epithelial to mesenchymal transition, and stemness.[13]

We have established anti-PDPN mAbs against 20 species,[14–31] which can be used for flow cytometry and immunohistochemistry. These mAbs are expected to contribute not only to the research of each animal but also to pathogenic diagnosis. In a previous study, we established an anti-elephant PDPN (elePDPN) mAb PMab-295 (IgG_1, kappa) using the peptide immunization.[15] To clarify further characteristics of PMab-295, we performed epitope mapping using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Peptides

The elePDPN (accession No.: XM_010593105.2) peptide ($_{38}$-EGGMVIPGVEDNMV-$_{51}$) and 14 alanine-substituted peptides (Table 1) were synthesized by utilizing PEPScreen (Sigma-Aldrich Corp., St. Louis, MO).

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Table 1. Identification of the PMab-295 Epitope Using Alanine-Substituted elePDPN Peptides

Peptides	*Sequences*	*PMab-295*
WT (38–51 aa)	EGGMVIPGVEDNMV	+++
E38A	AGGMVIPGVEDNMV	+++
G39A	EAGMVIPGVEDNMV	+++
G40A	EGAMVIPGVEDNMV	+++
M41A	EGGAVIPGVEDNMV	+++
V42A	EGGMAIPGVEDNMV	−
I43A	EGGMVAPGVEDNMV	+
P44A	EGGMVIAGVEDNMV	−
G45A	EGGMVIPAVEDNMV	+++
V46A	EGGMVIPGAEDNMV	+++
E47A	EGGMVIPGVADNMV	−
D48A	EGGMVIPGVEANMV	+
N49A	EGGMVIPGVEDAMV	+++
M50A	EGGMVIPGVEDNAV	+++
V51A	EGGMVIPGVEDNMA	+++

+++, OD655≧0.5; +, 0.1≦OD655<0.3; −, OD655<0.1; aa, amino acids; PDPN, podoplanin.

Enzyme-linked immunosorbent assay

Synthesized elePDPN peptides were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA) at a concentration of 10 μg/mL for 30 minutes at 37°C. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc., Kyoto, Japan), wells were blocked with 1% bovine serum albumin (BSA)-containing PBST for 30 minutes at 37°C. The plates were incubated with 1 μg/mL of PMab-295, followed by peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Flow cytometry

CHO/2×RIEDL-elePDPN (CHO/elePDPN) cells[15] were harvested after a brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.) and washed with 0.1% BSA (Nacalai Tesque, Inc.) in PBS (Nacalai Tesque, Inc.). PMab-295 (1 μg/mL) was incubated with each peptide (10 μg/mL) for 30 minutes at 4°C. CHO/elePDPN cells were treated with PMab-295+each peptide, and further treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Biotechnology Corp., Tokyo, Japan).

Immunohistochemical analysis

Cell blocks were produced using iPGell (Genostaff Co., Ltd., Tokyo, Japan)[23] and processed to make 4-μm-thick formalin-fixed paraffin-embedded (FFPE) cell sections. Those FFPE sections were directly autoclaved in a citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes, and were blocked using the SuperBlock T20 (PBS) blocking buffer (Thermo Fisher Scientific, Inc.). PMab-295 (1 μg/mL) was incubated with each peptide (10 μg/mL) in the blocking buffer for 1 hour at room temperature.

The tissue sections were incubated with PMab-295 (1 μg/mL)+each peptide for 1 hour at the room temperature, and then treated with the Envision+Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3,3′-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Results

Epitope mapping of PMab-295 with alanine-substituted PDPN peptide

We previously established an anti-elePDPN mAb (PMab-295) by peptide immunization of elePDPN extracellular domain ($_{38}$-EGGMVIPGVEDNMV-$_{51}$).[14] To reveal the binding epitope of PMab-295, we synthesized 14 alanine-substituted

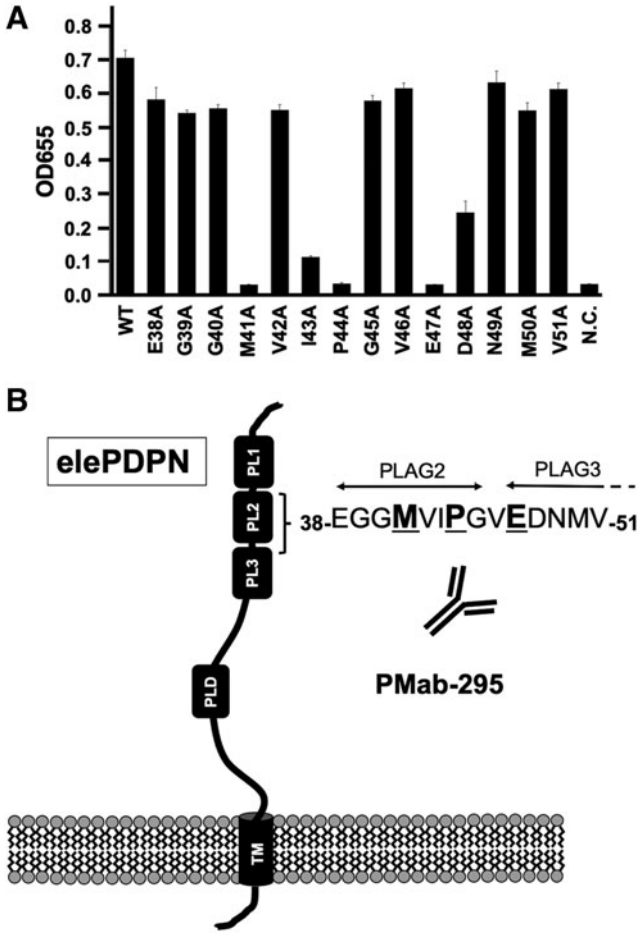

FIG. 1. Determination of the PMab-295 epitope of elePDPN by ELISA using alanine-substituted PDPN peptides. **(A)** The alanine-substituted elePDPN peptides were immobilized on immunoplates. The plates were incubated with PMab-295 (1 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of elePDPN and the PMab-295 epitope. The PMab-295 epitope involves Met41, Pro44, and Glu47 of elePDPN. ELISA, enzyme-linked immunosorbent assay; PDPN, podoplanin; PLAG, platelet aggregation-stimulating; PLD, PLAG-like domain; TM, transmembrane domain.

peptides of elePDPN (Table 1). PMab-295 exhibited reaction with E38A, G39A, G40A, V42A, I43A, G45A, V46A, D48A, N49A, M50A, V51A, and wild type (WT, 38-51 aa) (Fig. 1A). In contrast, PMab-295 did not react with M41A, P44A, and E47A (Fig. 1), indicating that Met41, Pro44, and Glu47 are included in the critical epitope of PMab-295. The results are summarized at Table 1. Figure 1B shows the schematic illustration of elePDPN and the critical epitope of PMab-295.

Flow cytometry using PMab-295 with alanine-substituted PDPN peptides

We next performed a peptide blocking assay using flow cytometry to confirm the importance of the PMab-295 epitope. As shown in Figure 2, PMab-295 reacted with the CHO/elePDPN cells. This reaction was almost completely neutralized by WT, E38A, G39A, G40A, V42A, G45A, V46A, N49A, M50A, and V51A. Furthermore, the reaction is partially neutralized by I43A and D48A. In contrast, M41A, P44A, and E47A never blocked the reaction of PMab-295 with CHO/elePDPN. These results confirmed that Met41, Pro44, and Glu47 of elePDPN are critical for PMab-295 detection by flow cytometry.

Immunohistochemistry using PMab-295 with alanine-substituted PDPN peptides

We also performed a blocking assay using immunohistochemical analysis. PMab-295 stained the CHO/elePDPN cells-embedded section (Fig. 3B), which was completely neutralized by WT (Fig. 3C), but not by M41A, P44A, and E47A (Fig. 3D, E), respectively. These results also confirmed that Met41, Pro44, and Glu47 of elePDPN are critical for PMab-295 detection by immunohistochemical analysis.

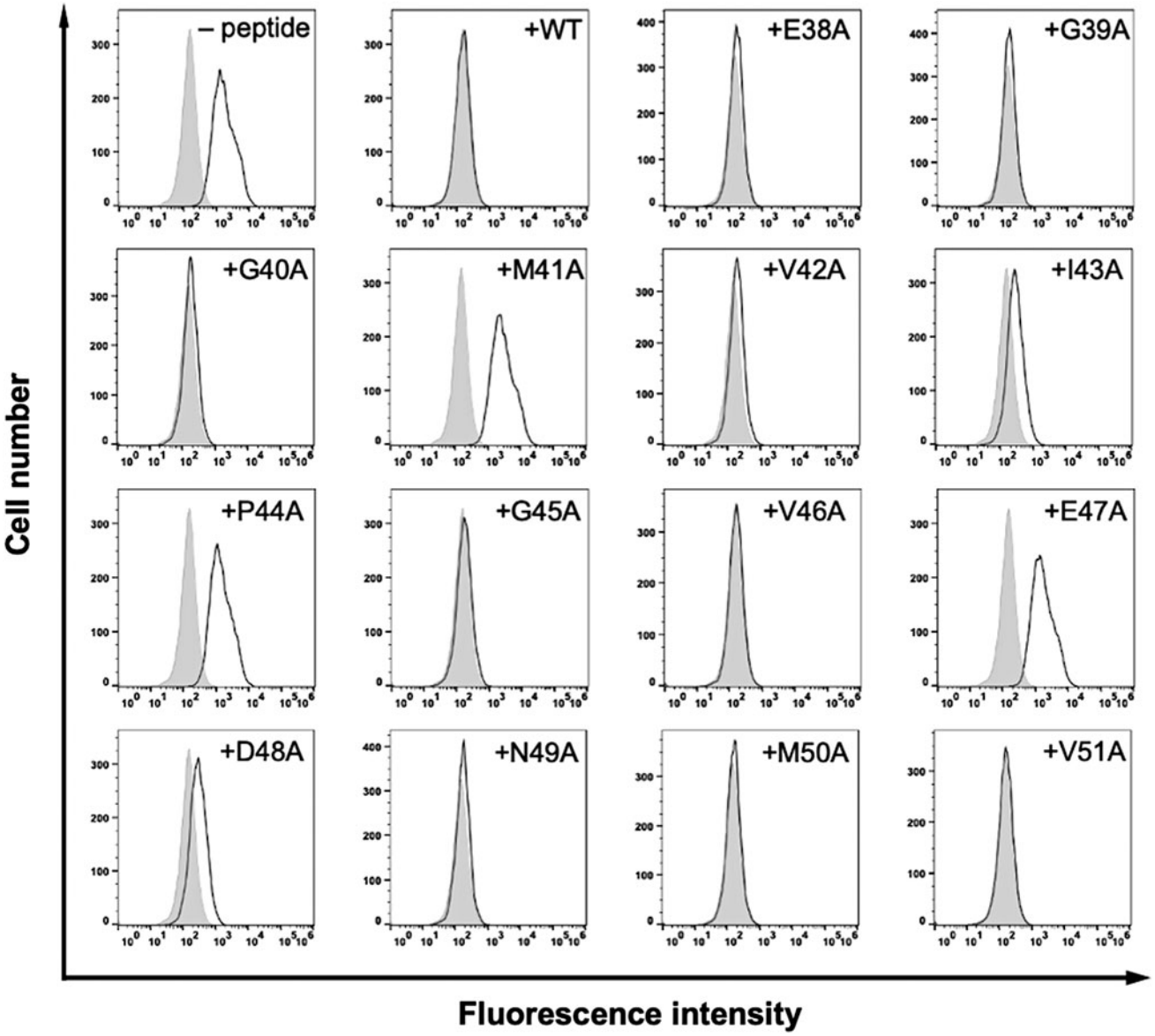

FIG. 2. Flow cytometry using PMab-295 and peptides of elePDPN. PMab-295 (1 μg/mL) plus the alanine-substituted peptides (10 μg/mL), or control (0.1% DMSO in PBS, –peptide) was reacted with CHO/elePDPN cells for 30 minutes at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Filled gray represents the negative control (PBS). DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.

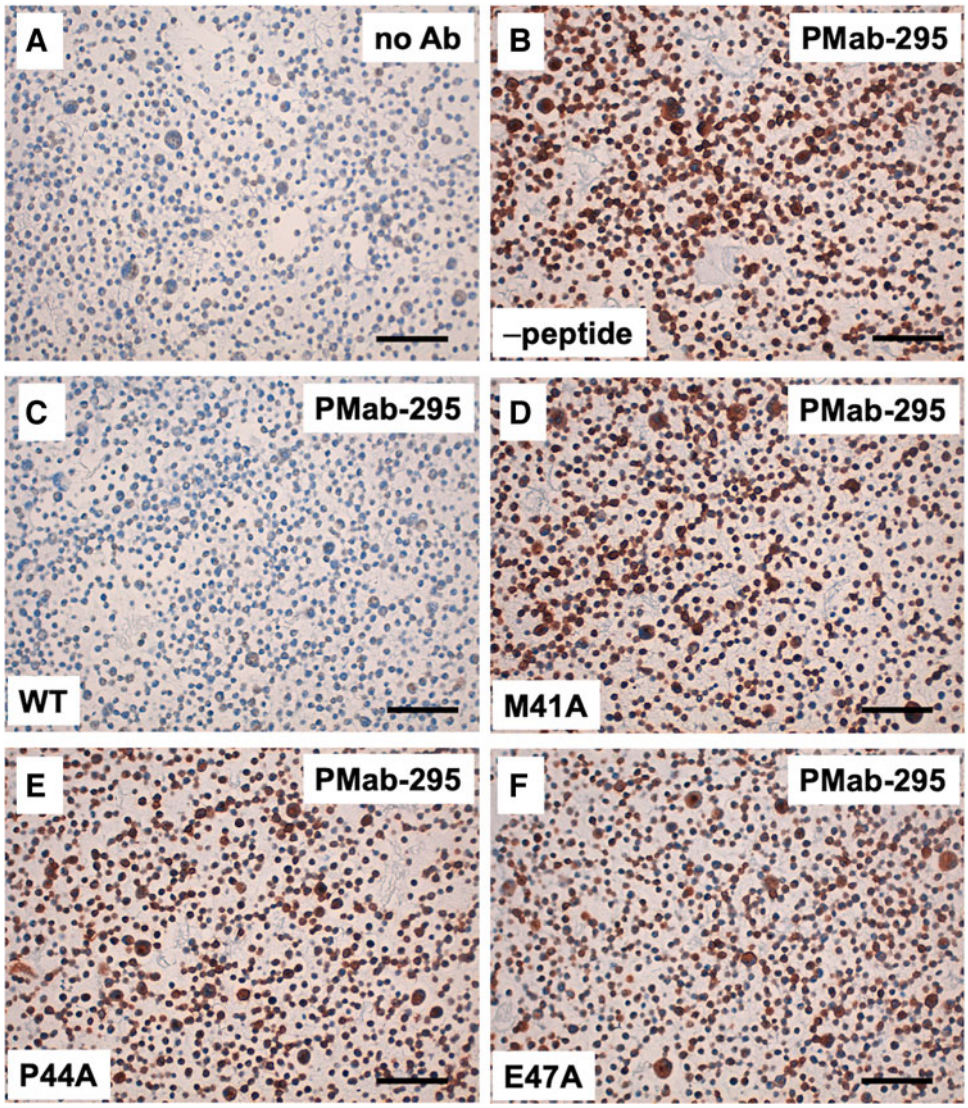

FIG. 3. Immunohistochemical analyses using PMab-295 and peptides of elePDPN. The FFPE sections of CHO/elePDPN cells were incubated with PMab-295 (1 μg/mL) plus the alanine-substituted peptides (10 μg/mL), or control (0.1% DMSO in blocking buffer, –peptide), followed by that with the Envision+Kit. **(A)** no antibody (Ab), **(B)** PMab-295, **(C)** PMab-295 + WT peptide, **(D)** PMab-295 + M41A peptide, **(E)** PMab-295 + P44A peptide, **(F)** PMab-295 + E47A peptide. Scale bar = 100 μm. DMSO, dimethyl sulfoxide; FFPE, formalin-fixed paraffin-embedded.

Discussion

PLAG domains of PDPN possess three tandem repeats of the ''EDxxVTPG'' consensus sequences.[4,9] In this study, we determined the critical epitope of PMab-295 as Met41, Pro44, and Glu47 of elePDPN between PLAG2 and PLAG3 domains (Fig. 1B). The *O*-glycosylation of Thr in the PLAG3 or PLD (also named as PLAG4 domain) has been reported to be essential for PDPN-induced platelet aggregation.[12,32] However, the PLAG2 domain of elePDPN is the ''EGGMVIPG'' sequence, which does not possess the consensus Thr residue, indicating that the PMab-295 recognition would not be affected by glycosylation.

We have not examined the cross-reactivity of PMab-295 to other species. We searched the conservation of ''MVIPGVE'' sequence to other species PDPN using standard protein BLAST (the Basic Local Alignment Search Tool, NCBI). However, there is no species, which possess the corresponding three amino acids, suggesting that PMab-295 specifically recognizes elephant PDPN.

As shown in Figure 3, PMab-295 is useful for immunohistochemistry of FFPE CHO/elePDPN cells. However, we could not obtain elephant tissues and confirm the staining patterns using PMab-295. We showed that PMab-295 and its blocking peptides can be used in immunohistochemistry (Fig. 3). Therefore, the use of PMab-295 with the blocking peptides would help the identification of PDPN-expressing cells on elephant tissues. Recently, immunohistochemical analyses of platelet endothelial cell adhesion molecules-1 and von Willebrand factor have been reported in elephant endotheliotropic herpesvirus-hemorrhagic disease in young Asian elephants (*Elephas maximus*).[33–36]

Furthermore, the reproductive tract neoplasia in adult female Asian elephant was reported in the United States.[37]

PDPN is overexpressed in many human tumors including squamous cell carcinomas,[38–41] malignant gliomas,[42–52] sarcomas,[53,54] and malignant mesotheliomas.[55–61] Therefore, the analysis of PDPN-positive cells on elephant tissues and PDPN-expressing carcinomas by PMab-295 will help the elucidation of the pathogenesis of the diseases.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported in part by the Japan Agency for Medical Research and Development (AMED) under Grant Numbers to Y.K. JP22ama121008, JP22am0401013, JP22bm1004001, JP22ck0106730, and JP21am0101078.

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Received: April 21, 2022
Accepted: July 3, 2022