

Development and Characterization of Anti-Sheep Podoplanin Monoclonal Antibodies PMab-253 and PMab-260

Mika K. Kaneko,¹ Masato Sano,¹ Junko Takei,¹ Teizo Asano,¹ Yusuke Sayama,¹
 Hideki Hosono,¹ Atsushi Kobayashi,² Satoru Konnai,^{3,4} and Yukinari Kato^{1,5}

Anti-podoplanin (PDPN) monoclonal antibodies (mAbs) are needed as markers for lymphatic endothelial cells or type I alveolar cells in immunohistochemical analyses. We have developed anti-PDPN mAbs for many species, including humans, mice, rats, rabbits, dogs, cats, bovines, pigs, Tasmanian devils, alpacas, tigers, whales, goats, horses, and bears. This study develops and characterizes anti-sheep PDPN (sPDPN) mAbs using Cell-Based Immunization and Screening (CBIS) method. A RAP14 tag was added to the N-terminus of sPDPN, and an anti-RAP14 tag mAb (PMab-2) was used to measure the expression level of sPDPN in flow cytometry and Western blots. We immunized mice with sPDPN-overexpressing Chinese hamster ovary (CHO)-K1 (CHO/sPDPN) cells and screened mAbs against sPDPN using flow cytometry. Two of the mAbs, PMab-253 (immunoglobulin M [IgM], kappa) and PMab-260 (IgM, kappa), detected CHO/sPDPN cells specifically using flow cytometry and Western blots. Both PMab-253 and PMab-260 stained the renal glomerulus and Bowman's capsule, lymphatic endothelial cells of the lung and colon, and type I alveolar cells of the lung, suggesting PMab-253 and PMab-260, which were developed by CBIS method, can be applied to functional analyses of sPDPN. We also determined the binding epitope of PMab-253 and PMab-260 using flow cytometry. Analysis of sPDPN deletion mutants revealed that the N-terminus of the PMab-253 and PMab-260 epitope exists between amino acids 110 and 115 of sPDPN. Analysis of sPDPN point mutations revealed that the critical epitope of PMab-253 and PMab-260 includes Thr112 and Ser113 of sPDPN, indicating that the PMab-253 and PMab-260 epitope are independent of the platelet aggregation-stimulating (PLAG) domain or the PLAG-like domain of sPDPN.

Keywords: podoplanin, sheep, sPDPN, monoclonal antibody

Introduction

SHEEP ARE USED as experimental animals to study the immune system,^(1–3) development of the blood–brain barrier,⁽⁴⁾ and lymphopoiesis/lymphocyte recirculation,⁽⁵⁾ among other subjects. However, detailed morphological studies of sheep lymphatic vessels have not been performed due to the lack of specific markers for lymphatic endothelial cells. Podoplanin (PDPN) is a type I transmembrane *O*-glycoprotein, which is expressed in renal podocytes, the lymphatic endothelial cells of every organ, and type I alveolar cells of the lung.^(6,7) In morphological studies, PDPN is an important specific marker of lymphatic vessels.⁽⁷⁾ PDPN possesses platelet aggregation-stimulating (PLAG) domains⁽⁸⁾ and a PLAG-like domain,^(9–13) which bind to C-type

lectin-like receptor-2 (CLEC-2).⁽¹⁴⁾ The PDPN/CLEC-2 interaction induces platelet aggregation and separation of embryonic blood/lymphatic vessels.⁽¹⁵⁾

We previously developed monoclonal antibodies (mAbs) against human,⁽¹⁶⁾ mouse,⁽¹⁷⁾ rat,⁽¹⁸⁾ rabbit,⁽¹⁹⁾ dog,⁽²⁰⁾ cat,⁽²¹⁾ bovine,⁽²²⁾ pig,^(23,24) Tasmanian devil,⁽²⁵⁾ alpaca,⁽²⁶⁾ tiger,⁽²⁷⁾ whale,⁽²⁸⁾ goat,^(29,30) horse,^(31,32) and bear^(33,34) PDPNs using Cell-Based Immunization and Screening (CBIS) method.^(35–37) These mAbs have all proven useful in flow cytometry, Western blots, and immunohistochemical analyses. In this study, we describe the development of anti-sheep PDPN (sPDPN) mAbs using CBIS to stain lymphatic endothelial cells by immunohistochemistry. We also determined the binding epitope of anti-sPDPN mAbs using flow cytometry.

¹Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.

²Laboratory of Comparative Pathology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan.

³Laboratory of Infectious Diseases, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan.

⁴Department of Advanced Pharmaceuticals, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan.

⁵New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). Synthesized DNA (Eurofins Genomics KK, Tokyo, Japan) encoding sPDPN (Accession No.: XM_004013802.4) and an N-terminal RAP14 tag was subcloned into a pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The RAP14 tag comprised 14 amino acids (aa), (DMVNPGLDRIEDL) and is recognized by PMab-2 mAb⁽¹⁸⁾ and LpMab-7.⁽³⁸⁾ sPDPN

wild-type (WT) and mutation plasmids were transfected using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific, Inc., Waltham, MA). Stable transfectants were selected by limiting dilution and cultivation in medium containing 0.5 mg/mL of G418 (Nacalai Tesque, Inc., Kyoto, Japan).

CHO-K1, P3U1, CHO/sPDPN,⁽³⁹⁾ CHO/human PDPN (hPDPN),⁽⁸⁾ CHO/mouse PDPN (mPDPN),⁽⁸⁾ CHO/rat PDPN (rPDPN),⁽¹⁸⁾ CHO/rabbit PDPN (rabPDPN),⁽¹⁹⁾ CHO/dog PDPN (dPDPN),⁽²⁰⁾ CHO/bovine PDPN (bovPDPN),⁽²²⁾ CHO/cat PDPN (cPDPN),⁽²¹⁾ CHO/pig PDPN (pPDPN),⁽²⁴⁾ CHO/horse PDPN (horPDPN),⁽³¹⁾ CHO/tiger PDPN

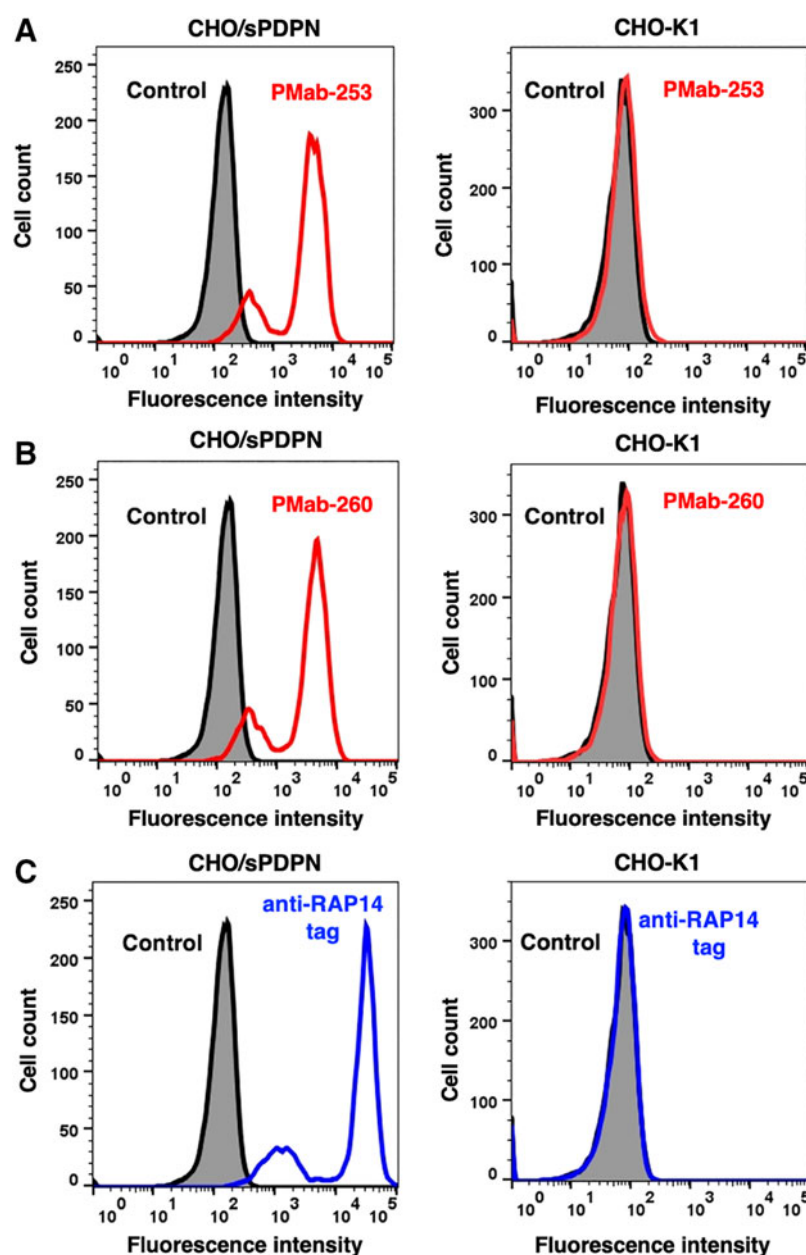


FIG. 1. Detection of sPDPN by flow cytometry using anti-sPDPN mAbs. CHO/sPDPN and CHO-K1 cells were treated with PMab-253 (A), PMab-260 (B), or anti-RAP14 tag (C) at a concentration of 1 μ g/mL or 0.1% BSA in PBS for 30 min, followed by incubation with secondary antibodies. PDPN, podoplanin; sPDPN, sheep PDPN; CHO, Chinese hamster ovary; BSA, bovine serum albumin; PBS, phosphate-buffered saline; mAbs, monoclonal antibodies.

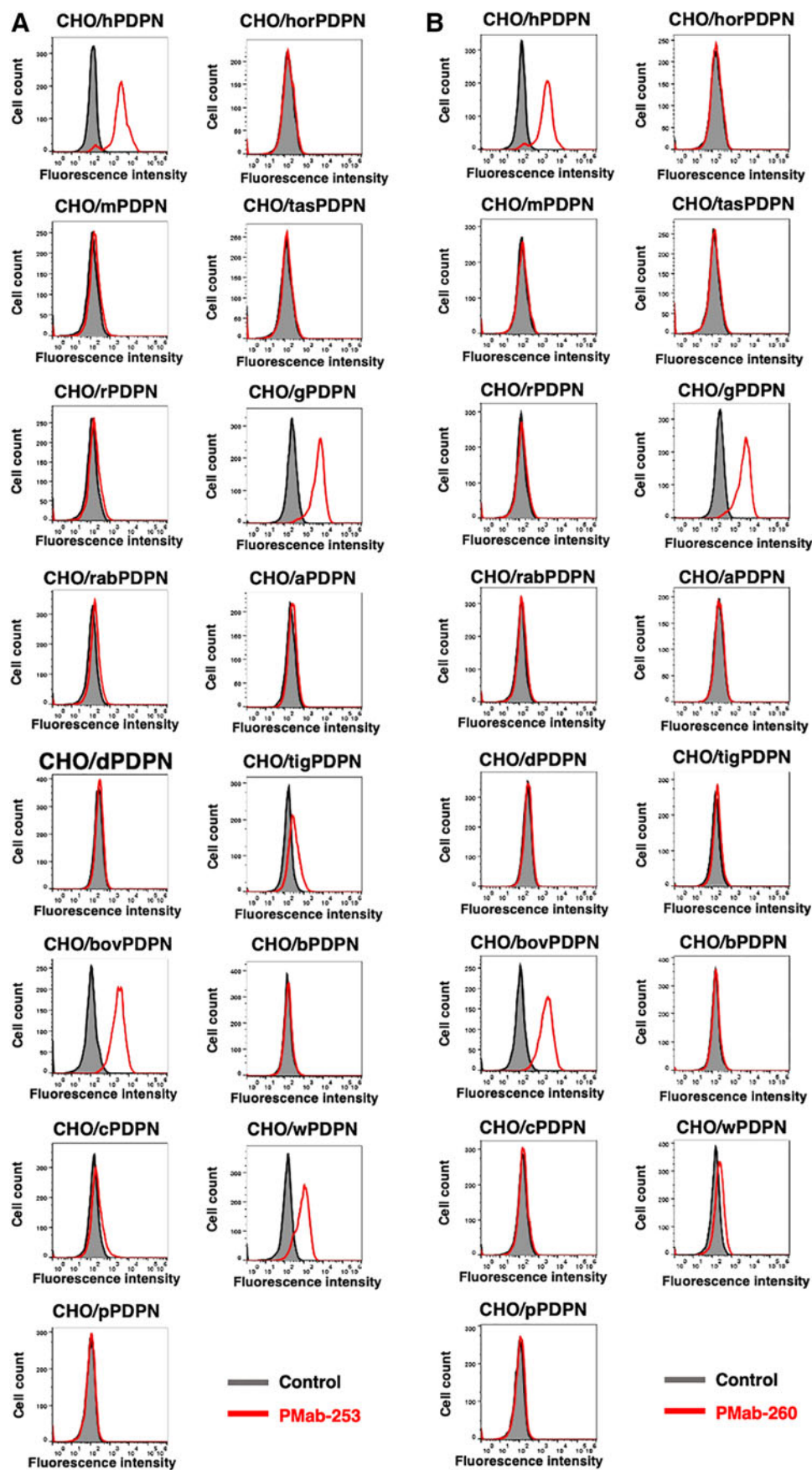


FIG. 2. Cross-reaction of anti-sPDPN mAbs with PDPNs of other species by flow cytometry. CHO-K1 cells transfected with PDPNs of other species were treated with PMAb-253 (A) or PMAb-260 (B) at a concentration of 5 μ g/mL or 0.1% BSA in PBS (gray) for 30 min, followed by incubation with secondary antibodies.

(tigPDPN),⁽²⁷⁾ CHO/alpaca PDPN (aPDPN),⁽²⁶⁾ CHO/bear PDPN (bPDPN),⁽²⁴⁾ CHO/Tasmanian devil PDPN (tasPDPN),⁽²⁴⁾ CHO/goat PDPN (gPDPN),⁽²⁷⁾ and CHO/whale PDPN (wPDPN)⁽²⁸⁾ were cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 0.25 μ g/mL of amphotericin B (Nacalai Tesque, Inc.). Cells were grown in a humidified incubator at 37°C with an atmosphere of 5% CO₂ and 95% air.

Animals

All animal experiments were performed according to relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2016Mda-153). Mice were monitored daily for health during the full 4-week duration of the experiment. A reduction of more than 25% of total body weight was defined as a humane endpoint. Mice were euthanized by cervical dislocation, and death was verified by respiratory and cardiac arrest. Female BALB/c mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under specific pathogen-free conditions.

Production of sPDPN mutants

Deletion mutants of the sPDPN sequence were produced using a HotStar HiFidelity polymerase kit (Qiagen, Inc., Hilden, Germany) with oligonucleotides. Substitutions of amino acids with alanine in the sPDPN sequence were produced 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).

Hybridoma production

To develop mAbs against sPDPN, we employed CBIS.^(21,35–37) Briefly, three BALB/c mice were immunized with CHO/sPDPN cells (1×10^8) by the intraperitoneal route together with the Inject Alum (Thermo Fisher Scientific, Inc.). The procedure included three additional immunizations followed by a final booster intraperitoneal injection administered 2 days before the harvest of spleen cells. Harvested spleen cells were subsequently fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN), and the hybridomas were grown in an RPMI medium supplemented with hypoxanthine, aminopterin, and thymidine for selection (Thermo Fisher Scientific, Inc.). Culture supernatants were screened by flow cytometry.

Flow cytometry

Cells were harvested following a brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). They were washed with 0.1% bovine serum albumin in phosphate-buffered saline (PBS) and treated with primary mAbs for 30 min at 4°C. The cells were then treated with Alexa Fluor 488-conjugated anti-mouse immunoglob-

ulin G (IgG, 1:2000; Cell Signaling Technology, Inc., Danvers, MA) or Oregon green anti-rat IgG (1:2000; Thermo Fisher Scientific, Inc.). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan).

Determination of binding affinity by flow cytometry

CHO/sPDPN was suspended in 100 μ L of serially diluted PMAb-253 and PMAb-260, and Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.) was added. Fluorescence data were collected using the EC800 cell analyzer (Sony Corp.). The dissociation constant (K_D) was calculated by fitting binding isotherms to built-in, one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Western blot analysis

Cell lysates (10 μ g) were boiled in a sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.). Proteins were then electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation) and transferred onto polyvinylidene difluoride membranes (Merck

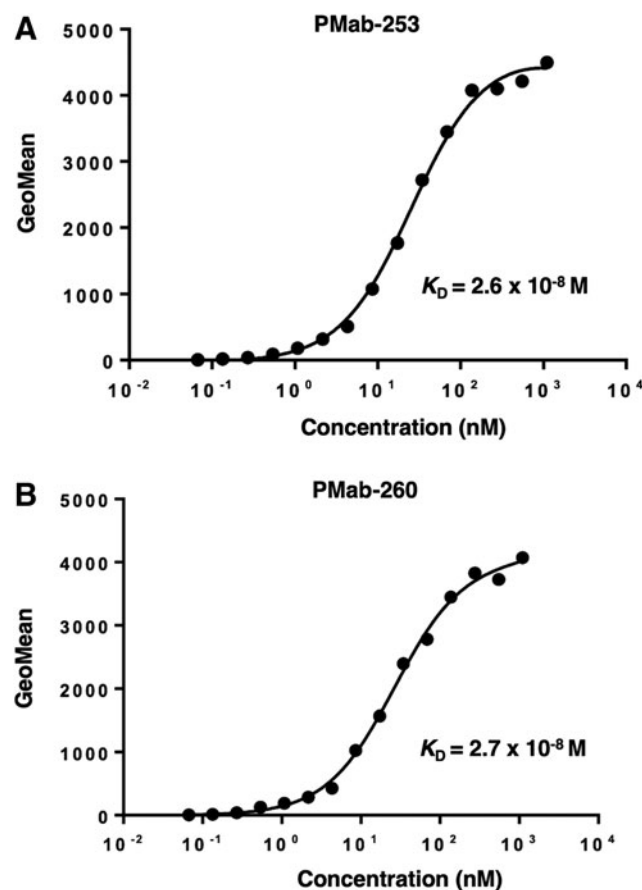


FIG. 3. Determination of binding affinity of anti-sPDPN mAbs. CHO/sPDPN was suspended in 100 μ L of serially diluted PMAb-253 (A) or PMAb-260 (B). Alexa Fluor 488-conjugated anti-mouse immunoglobulin G was then added. Fluorescence data were collected using the EC800 cell analyzer.

KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membranes were incubated with 5 μ g/mL of PMab-253, 5 μ g/mL of PMab-260, 1 μ g/mL of anti-RAP14 tag (clone: PMab-2),⁽¹⁸⁾ or 1 μ g/mL of anti- β -actin (clone AC-15; Sigma-Aldrich Corp., St. Louis, MO) and incubated with peroxidase-conjugated anti-mouse immunoglobulins (Agilent Technologies, Inc.; diluted 1:2000). The membranes were finally developed using the Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific, Inc.) or ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Immunohistochemical analyses

Normal sheep tissues from two sheep were collected after autopsies at Hokkaido University, fixed in 10% neutral-buffered formalin, and processed routinely to make formalin-fixed paraffin-embedded (FFPE) sections as in our previous study.⁽³⁹⁾ Histological sections 4- μ m thick were directly autoclaved in a citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 min. After blocking with SuperBlock T20 (PBS) blocking buffer (Thermo Fisher Scientific, Inc.), the sections were incubated with PMab-253 and PMab-260 (5 μ g/mL) for 1 h at room temperature and treated using an Envision+ kit (Agilent Technologies, Inc.) for 30 min. Color was developed using 3,3'-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 2 min, and counterstaining was performed with hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Results

Establishment of anti-sPDPN mAbs

To produce anti-sPDPN mAbs, we employed CBIS, using stable transfectants for both immunization and flow cytometric screening. Three mice were immunized with CHO/sPDPN cells, which overexpress sPDPN. Hybridomas were seeded into 96-well plates, and CHO/sPDPN-positive and CHO-K1-negative wells were selected. PMab-253 (immunoglobulin M [IgM], kappa) and PMab-260 (IgM, kappa) were selected using Western blotting and immunohistochemistry.

Flow cytometric analyses

We performed flow cytometry using PMab-253 and PMab-260 against CHO/sPDPN and CHO-K1. Both PMab-253 and PMab-260 recognized CHO/sPDPN, but not CHO-K1 (Fig. 1A, B). As a positive control, an anti-RAP14 tag mAb (PMab-2) detected CHO/sPDPN. The results indicate that PMab-253 and PMab-260 can detect sPDPN in flow cytometry analysis.

Next, the cross-reaction of PMab-253 and PMab-260 with the other PDPNs, which are overexpressed in CHO-K1 cells, was investigated. PMab-253 cross-reacted with hPDPN, bovPDPN, cPDPN, gPDPN, tigPDPN, and wPDPN (Fig. 2A). In contrast, PMab-260 cross-reacted with hPDPN, bovPDPN, gPDPN, and wPDPN (Fig. 2B).

We then assessed the apparent binding affinity of PMab-253 and PMab-260 with CHO/sPDPN using flow cytometry. The K_D of PMab-253 and PMab-260 for CHO/sPDPN was

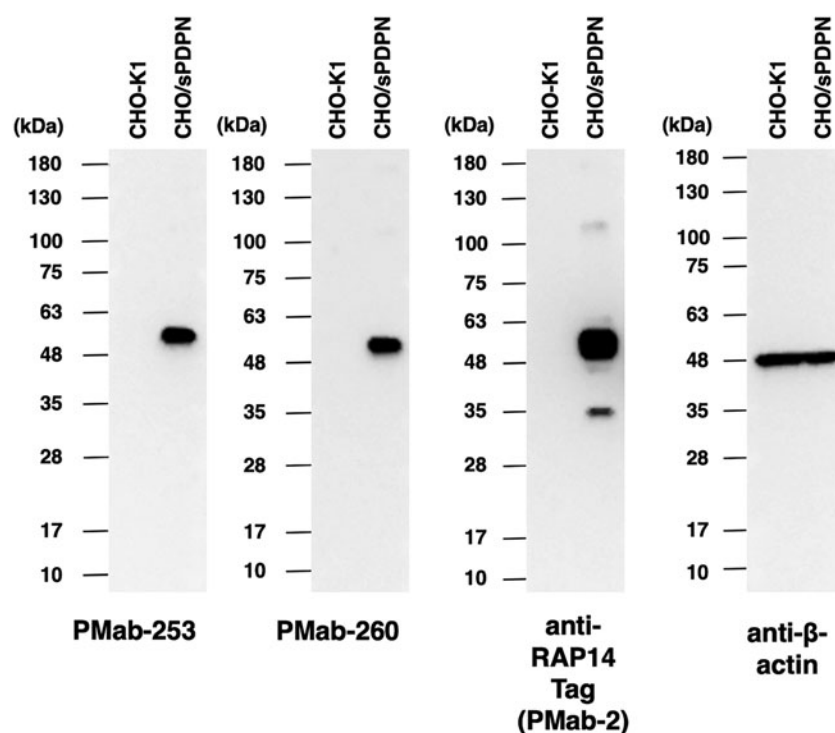


FIG. 4. Western blot analysis. Cell lysates of CHO-K1 and CHO/sPDPN (10 μ g) were electrophoresed and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with 5 μ g/mL of PMab-253, 5 μ g/mL of PMab-260, 1 μ g/mL of anti-RAP14 tag (PMab-2), and 1 μ g/mL of anti- β -actin and subsequently with peroxidase-conjugated anti-mouse immunoglobulins.

2.6×10^{-8} M and 2.7×10^{-8} M, respectively (Fig. 3), indicating that PMab-253 and PMab-260 possess moderate affinity for CHO/sPDPN cells.

Western blot analyses

We next investigated whether PMab-253 and PMab-260 can be applied to Western blot analysis by using cell lysates of CHO-K1 or CHO/sPDPN. As shown in Figure 4, PMab-253 and PMab-260 detected sPDPN as a 55-kDa band (N-terminal RAP14 tag + sPDPN) in CHO/sPDPN cell lysates, but these antibodies did not detect any band from CHO-K1 cells. Anti-RAP14 tag (PMab-2) as a positive control also detected a band of the same molecular weight from CHO/sPDPN. These results indicate that PMab-253 and PMab-260 can detect sPDPN in Western blot analysis.

Immunohistochemical analyses

We next investigated whether PMab-253 and PMab-260 can be applied to immunohistochemical analysis using FFPE sheep sections. For this assessment, we selected normal sheep

lung, kidney, and colon tissues, all of which reportedly express PDPN in other species, including humans,⁽¹⁶⁾ mice,⁽¹⁷⁾ rats,⁽¹⁸⁾ rabbits,⁽¹⁹⁾ dogs,⁽²⁰⁾ cats,⁽²¹⁾ bovines,⁽²²⁾ pigs,^(23, 24) Tasmanian devils,⁽²⁵⁾ alpacas,⁽²⁶⁾ tigers,⁽²⁷⁾ whales,⁽²⁸⁾ goats,^(29,30) horses,^(31,32) and bears.^(33,34) As shown in Figure 5A and B, PMab-253 stained the renal glomerulus and Bowman's capsule. Lymphatic endothelial cells (red arrow) of the colon were readily detected by PMab-253 (Fig. 5C, D). Furthermore, PMab-253 strongly stained type I alveolar cells and lymphatic endothelial cells (red arrow) of the lung (Fig. 5E, F). Similarly, PMab-260 strongly stained the renal glomerulus (Fig. 6A, B), lymphatic endothelial cells (red arrow) of colon (Fig. 6C, D), and type I alveolar cells and lymphatic endothelial cells (red arrow) of the lung (Fig. 6E, F).

Epitope mapping

Eleven deletion mutants of sPDPN in CHO-K1 cells were produced as follows: dN30, aa 30–168; dN40, aa 40–168; dN50, aa 50–168; dN60, aa 60–168; dN70, aa 70–168; dN80, aa 80–168; dN100, aa 100–168; dN105, aa 105–168; dN110,

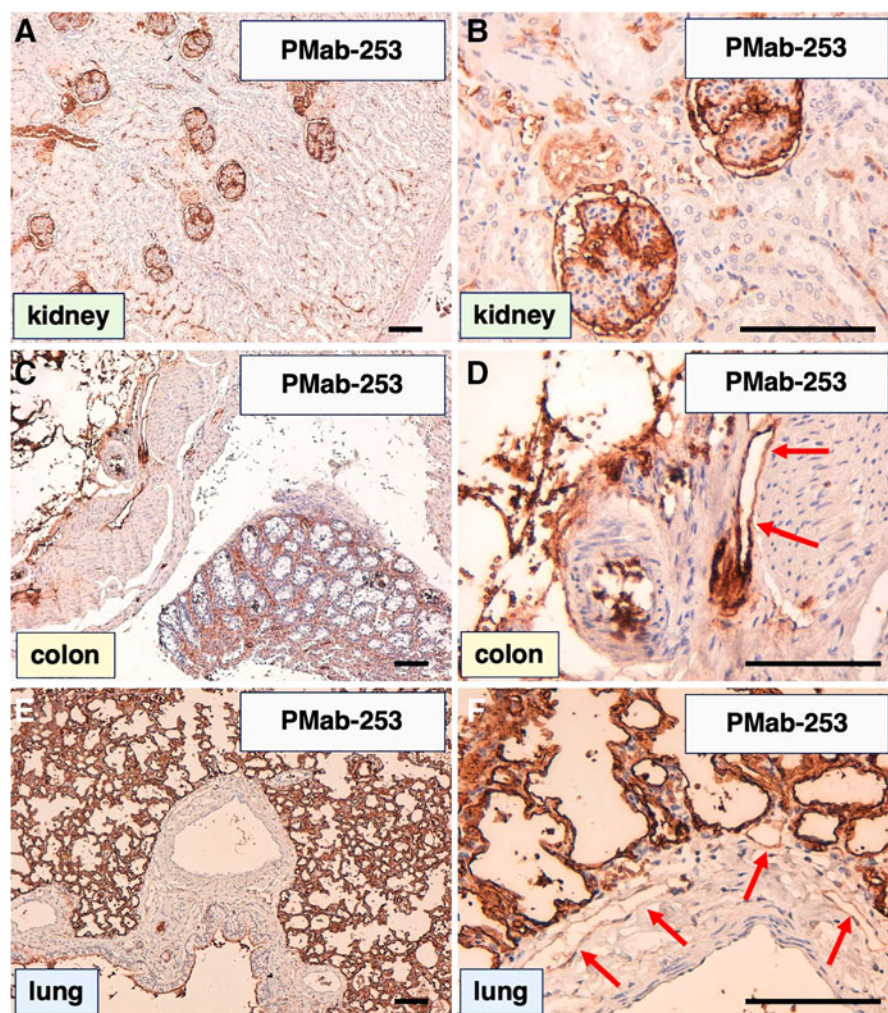


FIG. 5. Immunohistochemical analyses of sheep tissues using PMab-253. Formalin-fixed paraffin-embedded tissue sections of sheep kidneys (A, B), sheep colons (C, D), and sheep lungs (E, F) were incubated with 5 μg/mL of PMab-253, followed by analysis with the Envision+ kit. Red arrows show lymphatic endothelial cells. Scale bar = 100 μm.

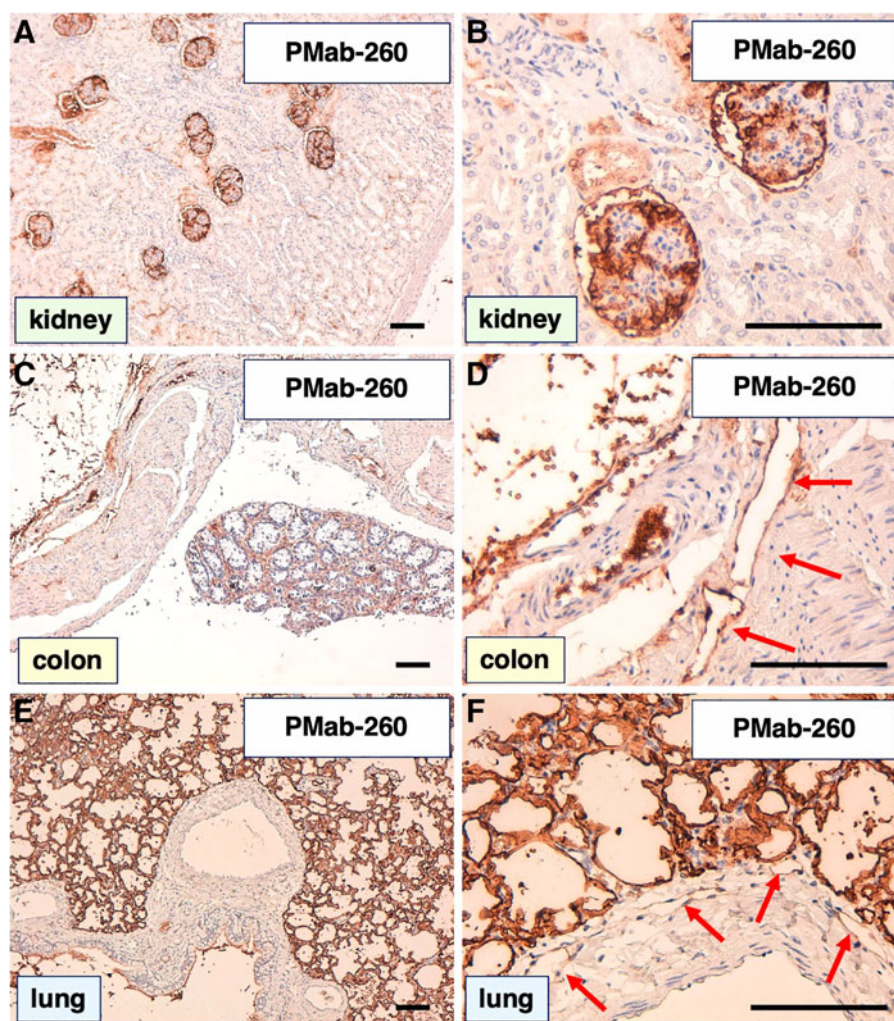


FIG. 6. Immunohistochemical analyses of sheep tissues using PMab-260. Formalin-fixed paraffin-embedded tissue sections of sheep kidneys (**A**, **B**), sheep colons (**C**, **D**), and sheep lungs (**E**, **F**) were incubated with 5 μ g/mL of PMab-260, followed by analysis with the Envision+ Kit. Red arrows show lymphatic endothelial cells. Scale bar = 100 μ m.

aa 110–168; dN115, aa 115–168; dN120, aa 120–168; or WT sPDPN, aa 27–168. All deletion mutants and WTs containing the N-terminal RAP14 tag were recognized by PMab-2 (an anti-RAP14 tag mAb), indicating that the expression level of each construct was high (Fig. 7A). PMab-253 recognized dN30, dN40, dN50, dN60, dN70, dN80, dN100, dN105, and dN110, but did not react with dN115 and dN120 (Fig. 7B), suggesting that the N-terminus of the PMab-253 epitope is between aa 110 and 115 of sPDPN. Similarly, PMab-260 recognized dN30, dN40, dN50, dN60, dN70, dN80, dN100, dN105, and dN110, but did not react with dN115 and dN120 (Fig. 7C), suggesting that the N-terminus of the PMab-260 epitope is also between aa 110 and 115 of sPDPN.

Next, we obtained a series of point mutants of sPDPN: V105A, H106A, T107A, H108A, V109A, P110A, A111G, T112A, S113A, H114A, S115A, and Q116A. PMab-2 reacted with all point mutants (Fig. 8A), but PMab-253 reacted less with T112A and S113A (Fig. 8B), indicating that Thr112 and Ser113 of sPDPN are essential for PMab-253 binding. PMab-260 reacted with T112A weakly, and did not react with S113A (Fig. 8C), also indicating that Thr112 and Ser113 of

sPDPN are essential for PMab-260 binding. The epitope of PMab-253 and PMab-260 is illustrated in Figure 9A and B, respectively.

Discussion

We previously investigated possible cross-reactions between anti-PDPN mAbs and sPDPN.⁽³⁹⁾ Flow cytometry analysis demonstrated that an anti-bovPDPN mAb (clone PMab-44)⁽²²⁾ reacted with CHO/sPDPN. Other anti-PDPN mAbs such as anti-mPDPN mAb (PMab-1),⁽¹⁷⁾ anti-rPDPN mAb (PMab-2),⁽¹⁸⁾ anti-rabPDPN mAb (PMab-32),⁽¹⁹⁾ anti-dPDPN mAb (PMab-38 and PMab-48), and anti-cPDPN mAb (PMab-52)⁽²¹⁾ did not react with CHO/sPDPN, indicating that only PMab-44 was useful for the detection of sPDPN. Expression of sPDPN was then investigated by immunohistochemical analyses using PMab-44. Although type I alveolar cells of sheep lungs were stained by PMab-44, the renal glomerulus and lymphatic endothelial cells were not stained by PMab-44.⁽³⁹⁾ As a result, novel anti-sPDPN mAbs for detecting lymphatic endothelial cells by immunohistochemical analysis are needed.

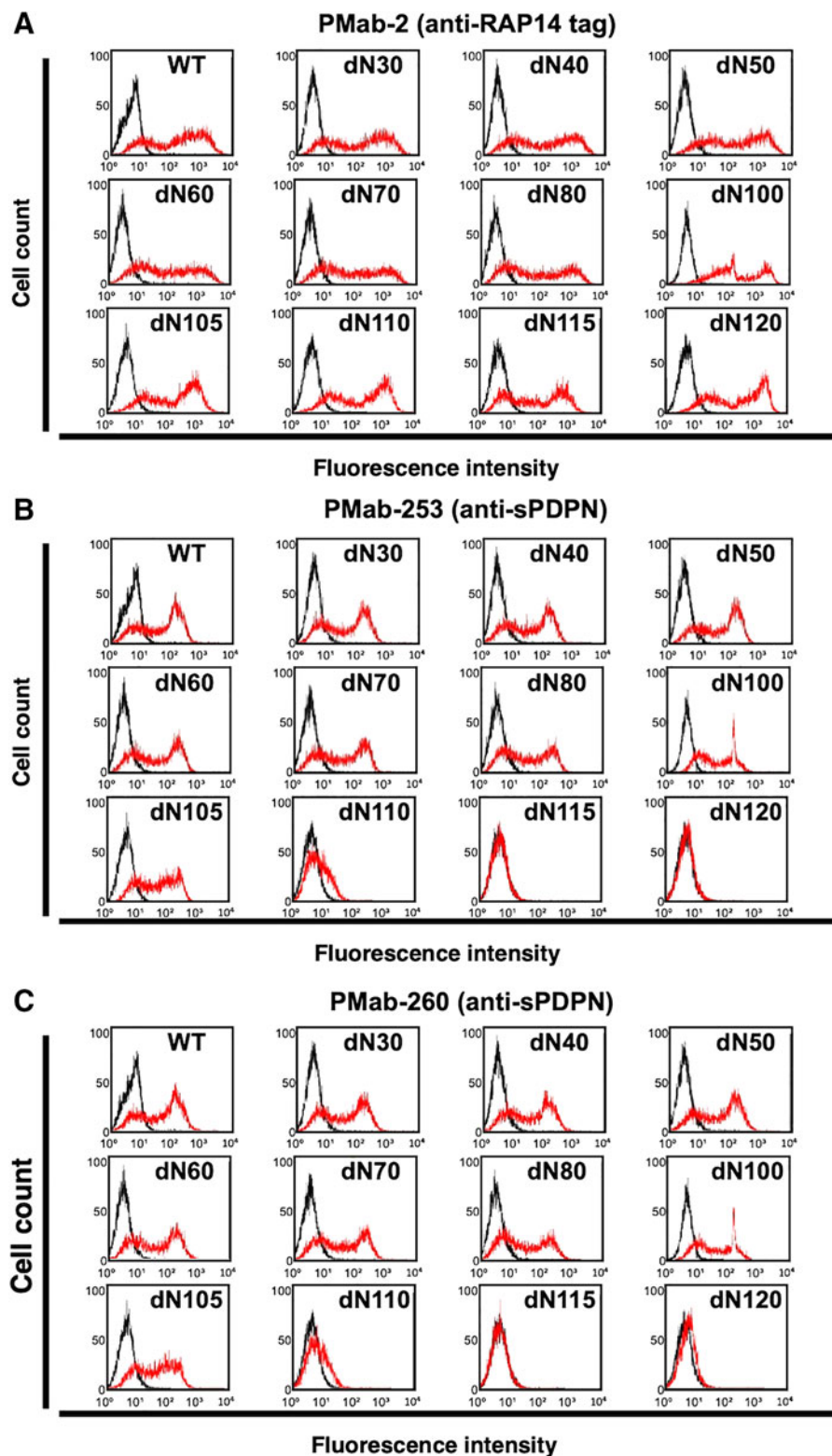


FIG. 7. Epitope mapping of PMAb-253 and PMAb-260 using deletion mutations. **(A)** Deletion mutants of sPDPN were analyzed using flow cytometry. Mutants were incubated with PMAb-2 (anti-RAP14 tag mAb; red line) or a buffer control (black line) for 30 min at 4°C, and then stained with secondary antibodies. **(B)** Deletion mutants of sPDPN were analyzed using flow cytometry. Mutants were incubated with PMAb-253 (anti-sPDPN mAb; red line) or a buffer control (black line) for 30 min at 4°C, and stained with secondary antibodies. **(C)** Deletion mutants of sPDPN were analyzed using flow cytometry. Mutants were incubated with PMAb-260 (anti-sPDPN mAb; red line) or buffer control (black line) for 30 min at 4°C and stained with secondary antibodies.

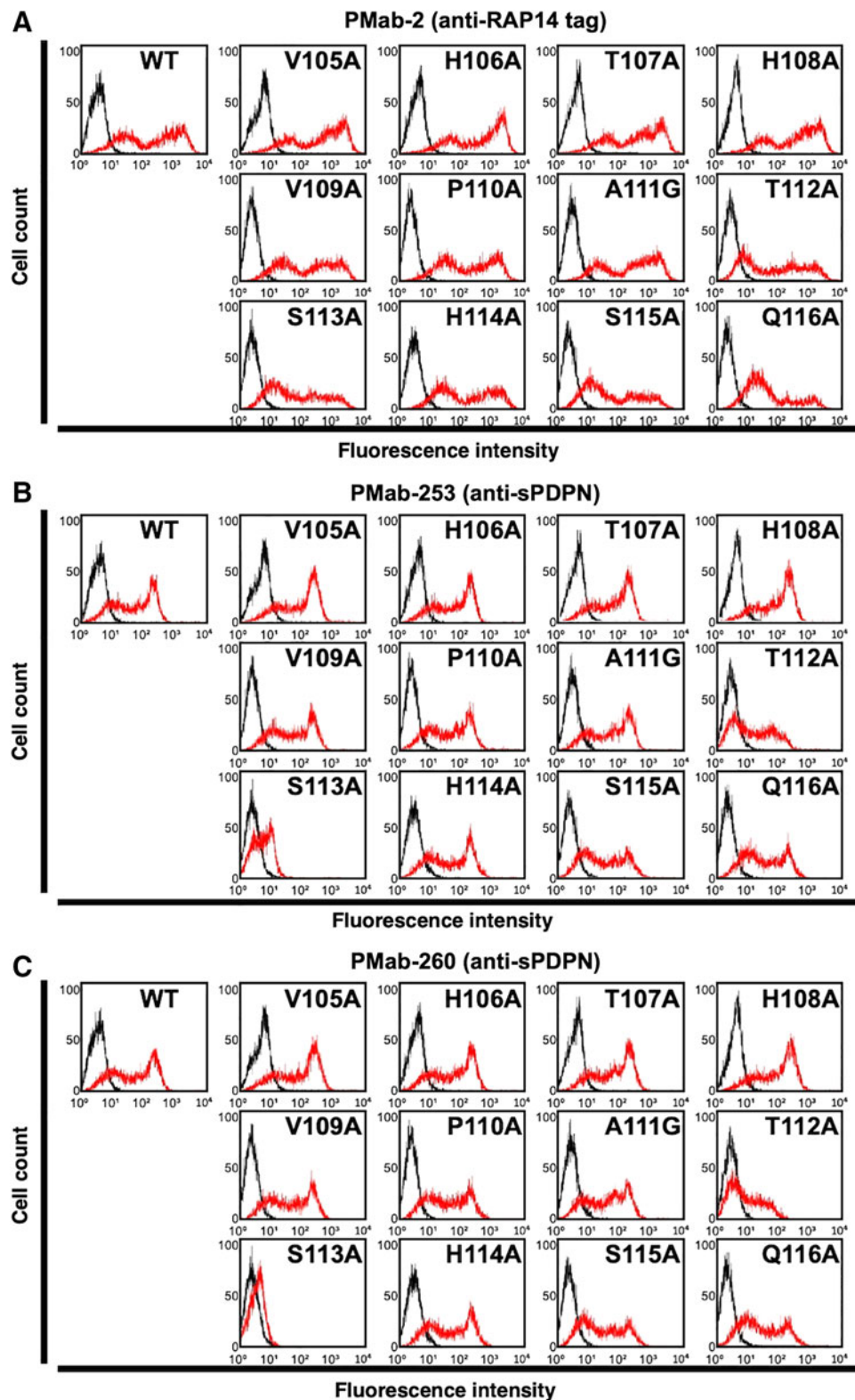


FIG. 8. Epitope mapping of PMAb-253 and PMAb-260 using point mutations. (A) Transient point mutants expressing V105A, H106A, T107A, H108A, V109A, P110A, A111G, T112A, S113A, H114A, S115A, and Q116A of sPDPN were incubated with PMAb-2 (anti-RAP14 tag; red line), or a buffer control (black line) for 30 min at 4°C, and stained with secondary antibodies. (B) Transient point mutants expressing V105A, H106A, T107A, H108A, V109A, P110A, A111G, T112A, S113A, H114A, S115A, and Q116A of sPDPN were incubated with PMAb-253 (anti-sPDPN mAb; red line) or a buffer control (black line) for 30 min at 4°C, followed by staining with secondary antibodies. (C) Transient point mutants expressing V105A, H106A, T107A, H108A, V109A, P110A, A111G, T112A, S113A, H114A, S115A, and Q116A of sPDPN were incubated with PMAb-260 (anti-sPDPN mAb; red line) or a buffer control (black line) for 30 min at 4°C and stained with secondary antibodies.

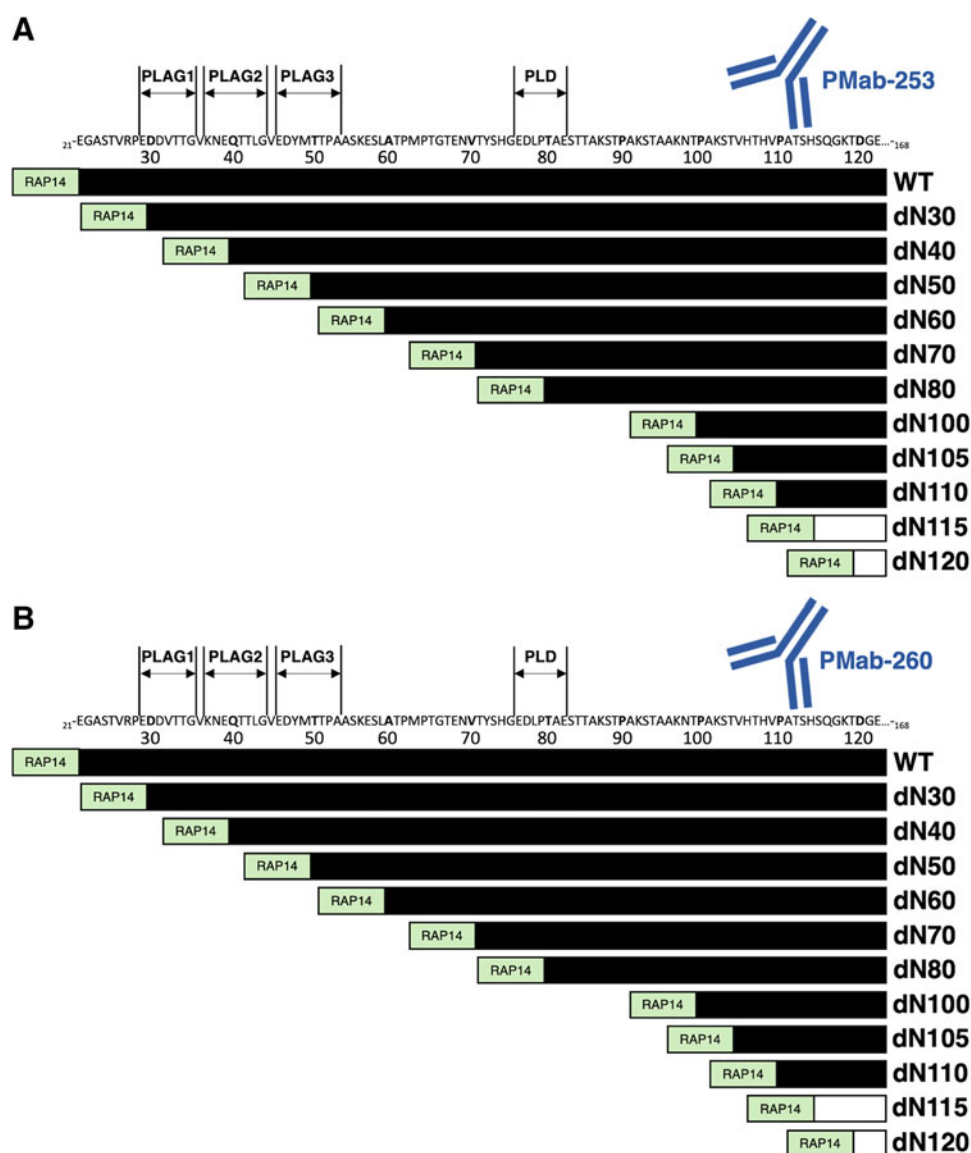


FIG. 9. Schematic illustration of the epitope recognized by PMAb-253 and PMAb-260. **(A)** Illustration of WT sPDPN and its 11 deletion mutants: dN30, dN40, dN50, dN60, dN70, dN80, dN100, dN105, dN110, dN115, and dN120. Black bars: positive reactions of PMAb-253. White bars: negative reactions of PMAb-253. **(B)** Illustration of WT sPDPN and its 11 deletion mutants: dN30, dN40, dN50, dN60, dN70, dN80, dN100, dN105, dN110, dN115, and dN120. Black bars: positive reactions of PMAb-260. White bars: negative reactions of PMAb-260. PLAG, platelet aggregation-stimulating; PLD, PLAG-like domain; WT, wild type.

In this study, we immunized mice with CHO/sPDPN cells, performed the first screening using only flow cytometry, and established two mAbs (PMAb-253 and PMAb-260). Although our previous study showed that PMAb-44 stained only type I alveolar cells and not lymphatic endothelial cells from sheep organs, PMAb-253 and PMAb-260 strongly stained lymphatic endothelial cells of the lung or colon (Figs. 5 and 6). Our recent study showed that one of the anti-bPDPN mAbs (clone PMAb-241) reacted with lymphatic endothelial cells, but not with type I alveolar cells of bear lungs.⁽³⁴⁾ The other anti-bPDPN mAb (clone PMAb-247) reacted not only with lymphatic endothelial cells but also type I alveolar cells and renal podocytes,⁽³³⁾ suggesting that the binding epitope of each mAb is critical to distinguishing PDPN-expressing lymphatic endothelial cells from other PDPN-expressing tissues. We therefore determined the binding epitope of PMAb-253 and

PMAb-260. Using a series of point mutants of sPDPN, Thr112 and Ser113 of sPDPN were determined to be essential for PMAb-253 and PMAb-260 binding. Until now, we have not found the binding epitope of anti-PDPN mAbs between aa 100 and aa 120 near the transmembrane domain of PDPN, indicating that the epitope of PMAb-253/PMAb-260 is novel. PMAb-253 and PMAb-260 may be advantageous for detailed morphological studies of sheep lymphatic vessels.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported, in part, by Japan Agency for Medical Research and Development (AMED) under Grant

Numbers: JP20am0401013 (Y.K.), JP20am0101078 (Y.K.), and JP20ae0101028 (Y.K.), and by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers: 17K07299 (M.K.K.), 19K07705 (Y.K.), and 20K16322 (M.S.).

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Address correspondence to:

Yukinari Kato
 New Industry Creation Hatchery Center
 Tohoku University
 2-1, Seiryomachi, Aoba-ku
 Sendai
 Miyagi 980-8575
 Japan

E-mail: yukinari-k@bea.hi-ho.ne.jp

Received: April 14, 2020

Accepted: June 20, 2020