

Characterization of Anti-Goat Podoplanin Monoclonal Antibody PMAb-235 Using Immunohistochemistry Against Goat Tissues

Junko Takei,^{1,2} Shunsuke Itai,^{1,2} Hiroyuki Harada,² Yoshikazu Furusawa,^{1,3,4} Takashi Miwa,⁴ Masato Fukui,⁴ Takuro Nakamura,¹ Masato Sano,¹ Yusuke Sayama,¹ Miyuki Yanaka,¹ Saori Handa,¹ Kayo Hisamatsu,¹ Yoshimi Nakamura,¹ Shinji Yamada,¹ Mika K. Kaneko,¹ and Yukinari Kato^{1,3}

Podoplanin (PDPN)/T1alpha is expressed on lymphatic endothelial cells, type I alveolar cells of the lungs, and podocytes of the kidney. PDPN possesses three platelet aggregation-stimulating (PLAG) domains (PLAG1, PLAG2, and PLAG3) of the N-terminus and the PLAG-like domains (PLDs). We previously reported an anti-goat PDPN (gPDPN) monoclonal antibody (mAb), PMAb-235, which was developed using the Cell-Based Immunization and Screening (CBIS) method. PMAb-235 is very useful in flow cytometry, Western blotting, and immunohistochemical analyses; however, the binding epitope of PMAb-235 remains to be elucidated. In this study, we investigated the epitopes of PMAb-235 using enzyme-linked immunosorbent assay and immunohistochemistry. The results revealed that the critical epitope of PMAb-235 produced by CBIS method is Arg75, Leu78, and Pro79 of gPDPN, which is included in PLD. The findings of our study can be applied to the production of more functional anti-gPDPN mAbs.

Keywords: podoplanin, PDPN, PMAb-235, epitope mapping

Introduction

PODOPLANIN (PDPN)/T1ALPHA IS A type I transmembrane glycoprotein, which is expressed in normal tissues, such as type I lung alveolar cells, renal corpuscles, and lymphatic endothelial cells.^(1,2) PDPN induces platelet aggregation through C-type lectin-like receptor-2 (CLEC-2) on platelets.^(1,3-9) The PDPN/CLEC-2 interaction facilitates the separation of embryonic blood/lymphatic vessels.⁽¹⁰⁾ The expression of human PDPN was reported in several malignant tumors, such as malignant brain tumors,⁽¹¹⁻¹⁴⁾ oral squamous cell carcinomas,⁽¹⁵⁾ malignant mesotheliomas,^(16,17) and lung cancers.⁽¹⁸⁾ PDPN expression has also been associated with malignant progression and cancer metastasis.^(6,11,19)

Because pathophysiological studies of alveolar cells of the lungs, podocytes of the kidneys, or lymphatic endothelial cells of the colon using anti-goat PDPN (gPDPN) mAbs are important in many disorders, we used the Cell-Based Immunization and Screening (CBIS) method and developed specific and sensitive monoclonal antibodies (mAbs) against the gPDPN of 168 amino acids to facilitate the immunohis-

tochemical analysis of paraffin-embedded tissue sections.⁽²⁰⁾ PMAb-235 recognized CHO/gPDPN cells, but showed no reaction with CHO-K1 cells, as assessed by flow cytometry. PMAb-235 cross-reacts with bovine PDPN. In contrast, PMAb-235 did not react with human, mouse, rat, rabbit, dog, cat, pig, horse, Tasmanian devil, sheep, alpaca, tiger, whale, or bear PDPNs. K_D of PMAb-235 for CHO/gPDPN cells was determined to be 1.5×10^{-8} , indicating a moderate affinity of PMAb-235 for CHO/gPDPN cells. The immunohistochemical analyses revealed that PMAb-235 strongly stained type I alveolar cells of lung, podocytes of kidney, and lymphatic endothelial cells of colon. This study aimed to determine the binding epitope of PMAb-235 to gPDPN using enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry.

Materials and Methods

Enzyme-linked immunosorbent assay

Synthesized gPDPN peptides using PEPscreen (Sigma-Aldrich Corp., St. Louis, MO) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific,

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

²Department of Oral and Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan.

³New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

⁴ZENOQA RESOURCE CO., LTD., Koriyama, Japan.

TABLE 1. DETERMINATION OF PMAB-235 EPITOPE BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Peptide	Sequence	PMab-235
pp21-40	EGASTVRPEDDVTGKNEQ	–
pp31-50	DVTGKNEQTTLGVEDYMT	–
pp41-60	TTLGVEDYMTTPAASKESLA	–
pp51-70	TPAASKESLATPMPTGTENV	–
pp61-80	TPMPTGTENVTHGHREDLPT	+++
pp71-90	THGHREDLPTAESTTAKSTP	–
pp81-100	AESTTAKSTPAESTPAKSTP	–
pp91-110	AESTPAKSTPAKSTVHTHVP	–
pp101-120	AKSTVHTHVPATSHSQGKTD	–
pp111-130	ATSHSQGKTDGEKPKSTQKG	–
pp121-135	GEKPKSTQKGGFSVG	–

+++ , OD655 \geq 0.6; – , OD655 < 0.1

Inc., Waltham, MA) at 10 μ g/mL for 30 minutes at 37°C. After blocking with SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), the plates were incubated with purified PMab-235 (1 μ g/mL), followed by a peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa Clara, CA) dilution of 1:2000. Enzymatic reactions were performed using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA). These reactions were performed at 37°C using a total sample volume of 50–100 μ L.

Immunohistochemical analyses

Goat tissues (lungs and colons) were previously collected in NIPPON ZENYAKU KOGYO CO., LTD. (Fukushima, Japan), and fixed in 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque, Inc., Kyoto, Japan).⁽²¹⁾ The tissues were processed to produce 4- μ m paraffin-embedded tissue sections, which were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. These tissue sections were then blocked with the SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), incubated with PMab-235 (0.01–1 μ g/mL) for 1 hour at room temperature, and treated using an Envision + Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Blocking assay using immunohistochemical analyses

Goat tissue sections were blocked with the SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.), incubated with PMab-235 (0.2 or 0.02 μ g/mL) or PMab-235 (0.2 or 0.02 μ g/mL) + peptides (1 μ g/mL) for 1 hour at room temperature, and treated using an Envision + Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed with DAB (Agilent Technologies, Inc.) for 2 minutes, and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

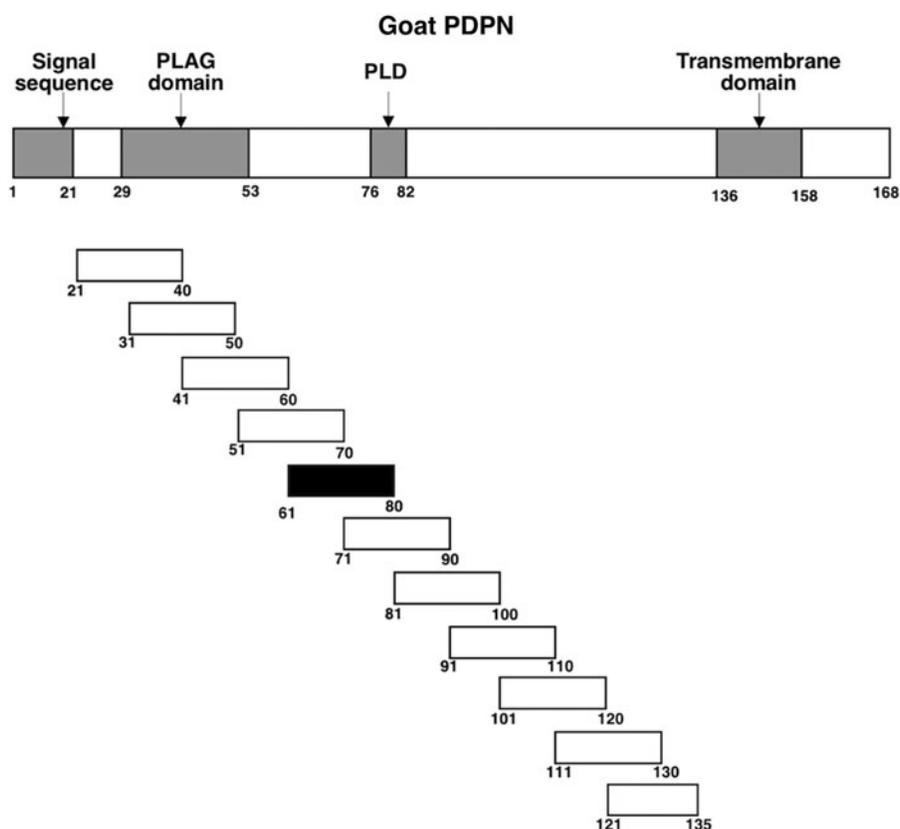


FIG. 1. Epitope mapping using synthetic peptides of gPDPN. Synthesized gPDPN peptides were immobilized at 10 μ g/mL, and were incubated with PMab-235, followed by secondary antibodies. Black bar: positive reaction by PMab-235. gPDPN, goat podoplanin.

Results

We first synthesized a series of gPDPN peptides, which are summarized in Table 1. Using ELISA, PMab-235 detected a 61–80 peptide (₆₁-TPMPTGTENVTHGHREDLPT-₈₀) of gPDPN, whereas it did not react with the other peptides: 21–40, 31–50, 41–60, 51–70, 71–90, 81–100, 91–110, 101–120, 111–130, and 121–135 peptides (Fig. 1 and Table 1).

We next moved on to the blocking assay to confirm the binding epitope of PMab-235. We first performed immunohistochemical analyses for goat lung tissues. PMab-235 showed very high reaction with type I alveolar cells of goat lungs in a dose-dependent manner (0.01–0.1 μg/mL; Fig. 2). Then, we performed a blocking assay using immunohistochemistry against goat lungs at a concentration of 0.02 μg/mL. PMab-235 reacted with type I alveolar cells of lung (Figs. 3A, B). These reactions were not neutralized by 21–40 peptide in lung (Figs. 3C, D). In contrast, these reactions were partially neutralized by 61–80 peptide in

lung (Figs. 3E, F), indicating that 61–80 peptide is an epitope of PMab-235.

PMab-235 also showed very high reaction with lymphatic endothelial cells of goat colons in a dose-dependent manner (0.1–1 μg/mL; Fig. 4), even at a low concentration of 0.1 μg/mL (Fig. 4G, H). We performed a blocking assay using immunohistochemistry against goat colons at a concentration of 0.2 μg/mL. PMab-235 reacted with lymphatic

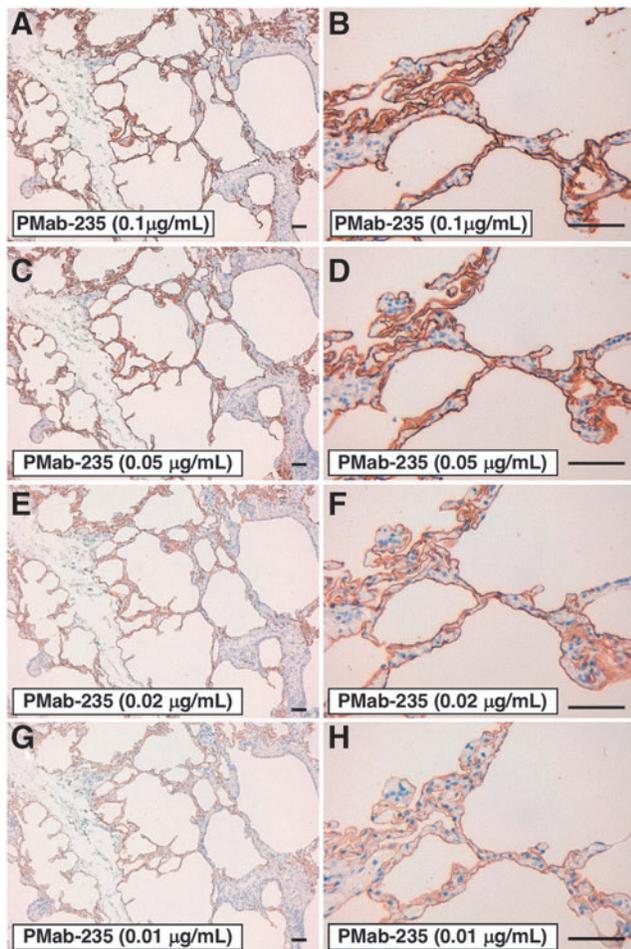


FIG. 2. Immunohistochemical analyses against goat lung tissues using PMab-235. Histological sections of the goat lungs were directly autoclaved in citrate buffer for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer, the sections were incubated with PMab-235 at a concentration of 0.1 μg/mL (A, B), 0.05 μg/mL (C, D), 0.02 μg/mL (E, F), and 0.01 μg/mL (G, H). Bar: 100 μm.

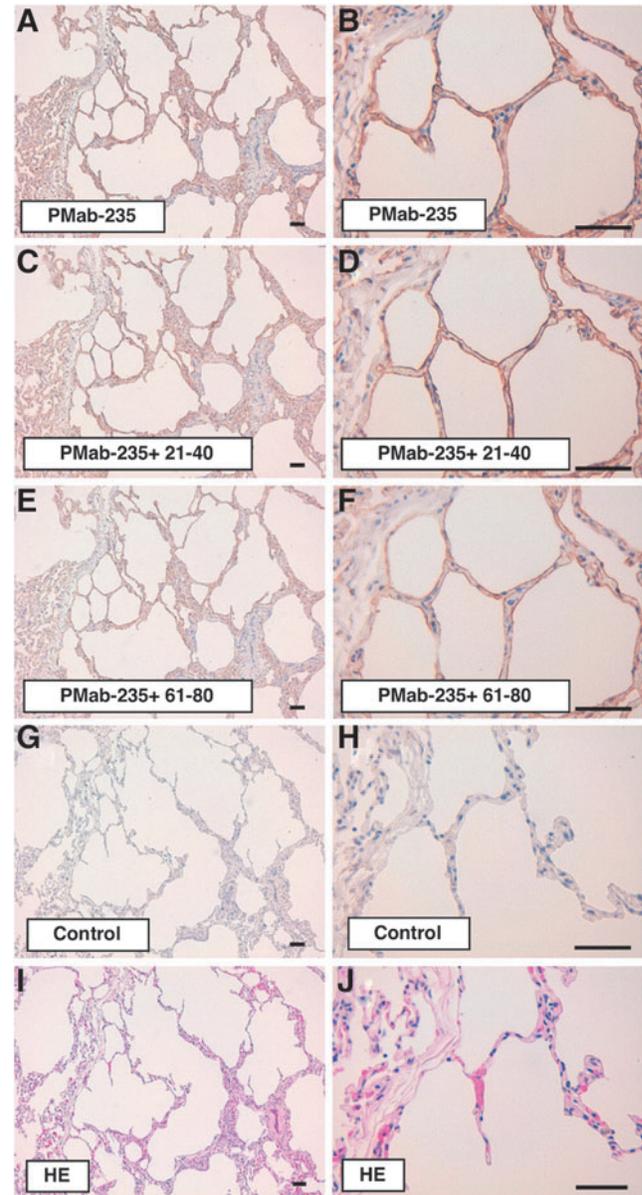


FIG. 3. Immunohistochemical analyses against goat lung tissues using PMab-235 and peptides of gPDPN. Histological sections of the goat lungs were directly autoclaved in citrate buffer for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer, the sections were incubated with (A, B) PMab-235 (0.02 μg/mL), (C, D) PMab-235 (0.02 μg/mL) + 21–40 (1 μg/mL), (E, F) PMab-235 (0.02 μg/mL) + 61–80 (1 μg/mL), or (G, H) control (blocking buffer), followed by treatment with the Envision+ Kit. (I, J). Bar: 100 μm. HE, hematoxylin and eosin.

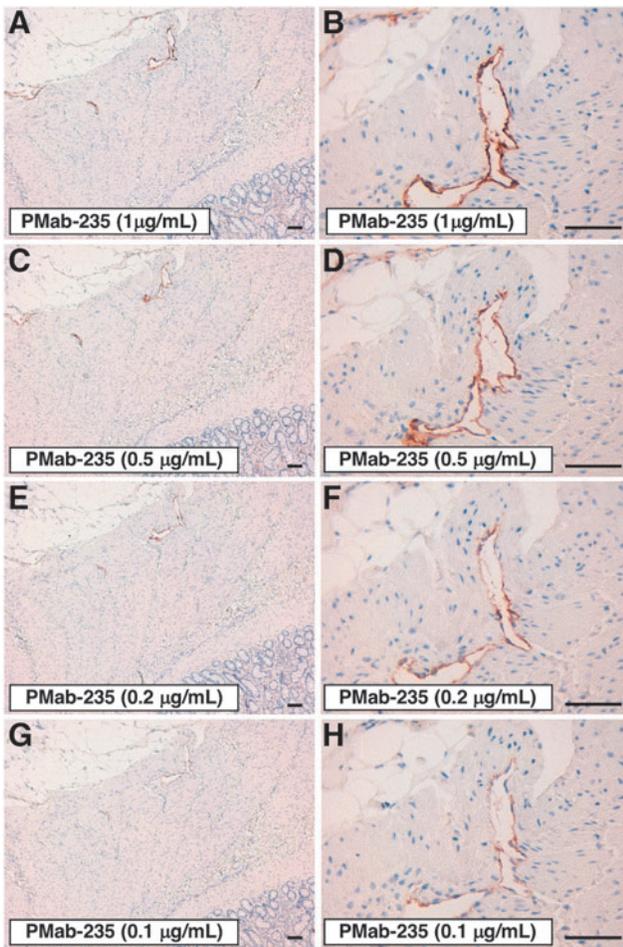


FIG. 4. Immunohistochemical analyses against goat colon tissues using PMAb-235. Histological sections of the goat colons were directly autoclaved in citrate buffer for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer, the sections were incubated with PMAb-235 at a concentration of 1 µg/mL (A, B), 0.5 µg/mL (C, D), 0.2 µg/mL (E, F), and 0.1 µg/mL (G, H). Bar: 100 µm.

endothelial cells of colons (Figs. 5A, B). These reactions were not neutralized by 21–40 peptide (Figs. 5C, D). In contrast, these reactions were completely neutralized by 61–80 peptide (Figs. 5E, F), also confirming that 61–80 peptide is an epitope of PMAb-235.

We then synthesized point mutants of 61–80 peptides (Table 2). Using ELISA, PMAb-235 detected T61A, P62A, M63A, P64A, T65A, G66A, T67A, E68A, N69A, V70A, T71A, H72A, G73A, H74A, E76A, D77A, and T80A. Conversely, it did not detect R75A, L78A, and P79A, indicating that R75A, L78A, and P79A are critical for PMAb-235 to detect gPDPN.

Discussion

We previously developed mAbs against human,⁽²²⁾ mouse,⁽²²⁾ rat,⁽²³⁾ rabbit,⁽²⁴⁾ dog,⁽²⁵⁾ cat,⁽²⁶⁾ bovine,⁽²⁷⁾ horse,^(28–30) and pig PDPNs.^(31,32) PDPN comprises three tandem-repeat of the “EDxxVTPG” sequences, which were defined to be platelet aggregation-stimulating (PLAG) do-

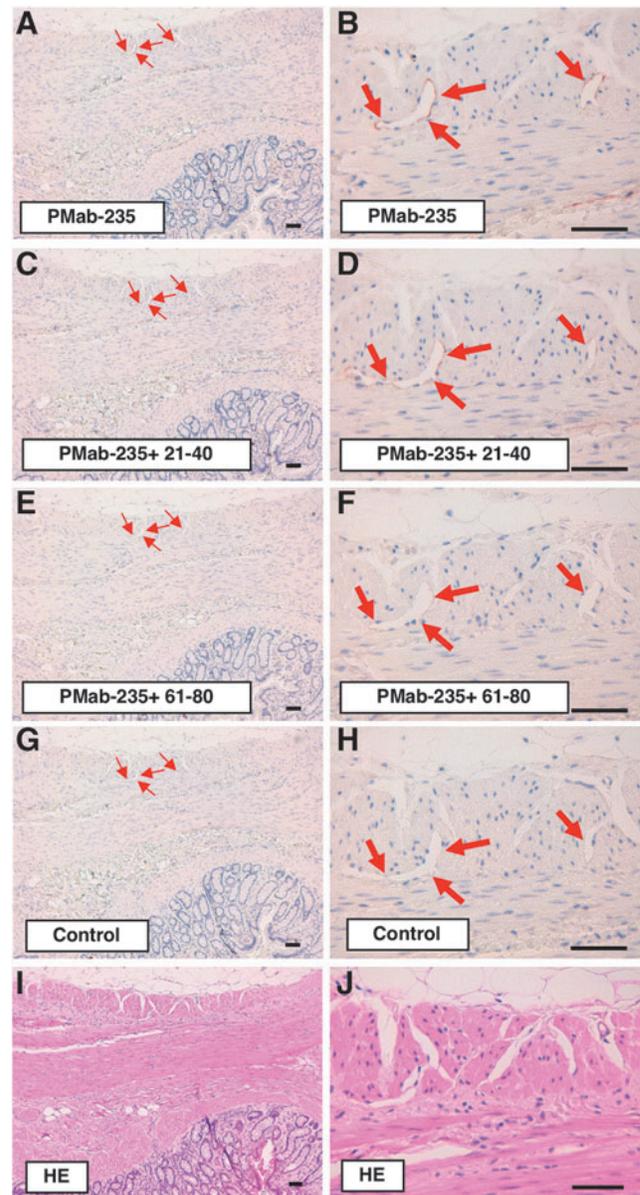


FIG. 5. Immunohistochemical analyses against goat colon tissues using PMAb-235 and peptides of gPDPN. Histological sections of the goat colons were directly autoclaved in citrate buffer for 20 minutes. After blocking with SuperBlock T20 (PBS) Blocking Buffer, the sections were incubated with (A, B) PMAb-235 (0.2 µg/mL), (C, D) PMAb-235 (0.2 µg/mL) + 21–40 (1 µg/mL), (E, F) PMAb-235 (0.2 µg/mL) + 61–80 (1 µg/mL), or (G, H) control (blocking buffer), followed by treatment with the Envision+Kit. (I, J) HE. Red arrows indicate lymphatic endothelial cells. Bar: 100 µm.

main (PLAG1, PLAG2, and PLAG3) of the N-terminus of the PDPN protein.⁽⁴⁾ There are also several PLAG-like domains (PLDs) of the “E(D/E)xx(T/S)xx” sequence. PLDs, one of which is also named as PLAG4, are reportedly important for PDPN–CLEC-2 interactions.⁽³³⁾ Almost all mAbs against PDPNs reportedly react with PLAG domains or PLDs.^(33,34) As summarized in Figure 6, PMAb-235 detected PLD of gPDPN.

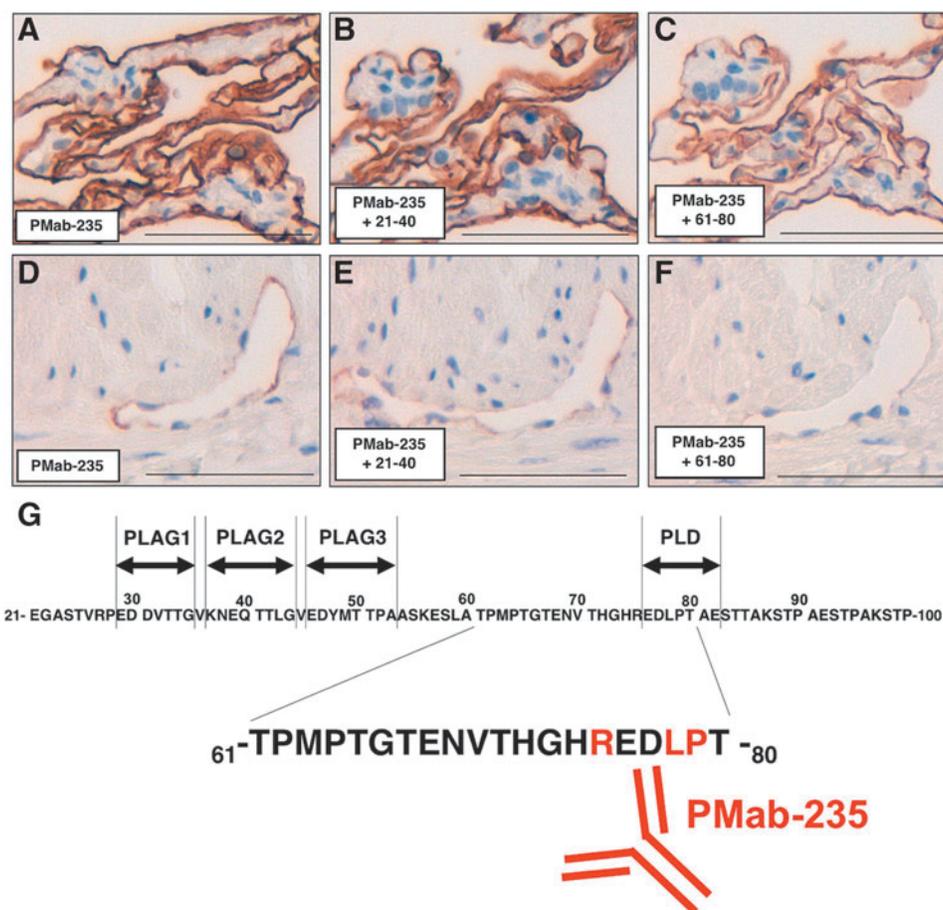


FIG. 6. Summary of PMab-235 epitope. (A–C) Immunohistochemical analyses against goat lung tissues using PMab-235 and peptides of gPDPN. Each picture is the enlargement of type I alveolar cells of Figure 3. (D–F) Immunohistochemical analyses against lymphatic endothelial cells of colon using PMab-235 and peptides of gPDPN. Each picture is the enlargement of lymphatic endothelial cells of Figure 5. Bar: 100 μm. (G) Schematic illustration of the epitope recognized by PMab-235. PLAG, platelet aggregation-stimulating; PLD, PLAG-like domain.

TABLE 2. DETERMINATION OF PMAB-235 EPITOPE BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Mutation	Sequence	PMab-235
T61A	APMPTGTENVTHGHREDLPT	+++
P62A	TAMPTGTENVTHGHREDLPT	+++
M63A	TPAPTGTENVTHGHREDLPT	+++
P64A	TPMATGTENVTHGHREDLPT	+++
T65A	TPMPAGTENVTHGHREDLPT	+++
G66A	TPMPTATENVTHGHREDLPT	+++
T67A	TPMPTGAENVTHGHREDLPT	+++
E68A	TPMPTGTANVTHGHREDLPT	+++
N69A	TPMPTGTEAVTHGHREDLPT	+++
V70A	TPMPTGTENATHGHREDLPT	+++
T71A	TPMPTGTENVAHGHREDLPT	+++
H72A	TPMPTGTENVTAGHREDLPT	+++
G73A	TPMPTGTENVTHAHREDLPT	+++
H74A	TPMPTGTENVTHGAREDLPT	+++
R75A	TPMPTGTENVTHGHAEDLPT	–
E76A	TPMPTGTENVTHGHRADLPT	+++
D77A	TPMPTGTENVTHGHREALPT	+++
L78A	TPMPTGTENVTHGHREDAPT	–
P79A	TPMPTGTENVTHGHREDLAT	–
T80A	TPMPTGTENVTHGHREDLPA	+++

+++ , OD655 ≥ 0.6; – , OD655 < 0.1

PMab-235 cross-reacted with bovine PDPN (bovPDPN).⁽²⁰⁾ The corresponding sequence of PMab-235 epitope “REDLPT” is also “REDLPT” about bovPDPN, which indicates 100% identity between gPDPN and bovPDPN. In the blocking assay using immunohistochemistry against goat lungs, the 61–80 peptide of goat PDPN did not completely block the PMab-235 binding to type I alveolar cells (Figs. 3E, F) because PMab-235 was developed using CBIS method, not by immunizing synthetic peptides, then the binding affinity of PMab-235 against type I alveolar cells might be higher than that for the 61–80 peptide.

Taken together, PLD of gPDPN was clarified to be advantageous epitopes for several applications, such as flow cytometry, Western blotting, and immunohistochemical analyses. These findings can be applied to the production of more functional anti-gPDPN mAbs.

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Author Disclosure Statement

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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: yukinarikato@med.tohoku.ac.jp

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