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Epitope Mapping of Monoclonal Antibody PMab-52 Against Cat Podoplanin

Yao-Wen Chang,[1] Mika K. Kaneko,[1] Shinji Yamada,[1] and Yukinari Kato[1,2]

The mucin-type membrane glycoprotein podoplanin (PDPN) is frequently overexpressed in numerous malignant cancers, including squamous cell carcinoma, germinal neoplasia, mesothelioma, lung cancer, oral cancer, and brain tumor. PDPN expression is strongly associated with cancer progression and poor prognosis. Furthermore, PDPN binds to C-type lectin-like receptor 2 (CLEC-2) on platelets, followed by PDPN-mediated platelet aggregation to facilitate tumor metastasis. We have previously reported a novel anti-cat PDPN (cPDPN) monoclonal antibody (mAb), PMab-52, which specifically detects cPDPN using flow cytometry analysis and successfully identifies cPDPN in feline squamous cell carcinomas. However, the specific binding epitope of cPDPN for PMab-52 remains unelucidated. In this study, a series of deletion or point mutants of cPDPN were utilized for investigating the binding epitopes of PMab-52 using flow cytometry and Western blotting. The findings of this study revealed that the critical epitopes of platelet aggregation-stimulating domain 4 (PLAG4) of cPDPN are responsible for the binding of PMab-52 to cPDPN.

Keywords: cat podoplanin, monoclonal antibody, epitope

Introduction

Podoplanin (PDPN/T1α/Aggrus), a highly *O*-glycosylated transmembrane glycoprotein,[1–3] is extensively distributed in normal tissues and cells, including kidney podocytes, lung alveolar cells, lymphatic endothelial cells, myofibroblasts, mesothelial cells, heart, and central nervous system.[4–8] Versatile physiological functions of PDPN have been reported to play crucial roles in embryonic cardiac development,[8,9] blood/lymphatic vessel separation,[10,11] and high endothelial venule integrity.[12] However, a high level of PDPN expression has been observed in several malignant tumors, including brain tumors,[13,14] oral cancers,[15] lung cancers,[16] melanomas,[17] mesotheliomas,[5] breast cancers,[18,19] central nervous system tumors,[20] and osteosarcomas.[21–23] Clinical data also indicated the expression of PDPN to be correlated with poor prognosis and tumor malignancy in lung carcinomas, oral squamous cell carcinomas, and breast cancers.[8,24–27] Furthermore, PDPN facilitates hematogenous metastasis by eliciting tumor cell-induced platelet aggregation response through its interaction with platelet C-type lectin-like receptor 2 (CLEC-2).[28–31] These pieces of evidence imply the importance of developing anti-PDPN monoclonal antibodies (mAbs) for cancer therapeutic treatment.

We have recently established a novel PMab-52 mAb, which specifically detects cat PDPN (cPDPN) using flow cytometry analysis and successfully recognizes cPDPN in feline squamous cell carcinomas.[32] However, the specific binding region of cPDPN for PMab-52 remains to be elucidated. In this study, we investigated the binding epitopes of PMab-52 by analyzing a series of deletion or point mutants of cPDPN using flow cytometry and Western blotting.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cPDPN mutation plasmids containing MAP tag were transfected into CHO-K1 cells using Lipofectamine LTX (Thermo Fisher Scientific, Inc., Waltham, MA). Transiently transfected cells with delete/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.

Production of cPDPN mutants

The cDNA of cPDPN was subcloned into a pCAG vector (Wako Pure Chemical Industries Ltd., Osaka, Japan), and an

[1]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[2]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

MAP tag was added at the N-terminus. Deletion mutants of cPDPN sequence were performed using a HotStar HiFidelity PCR (Qiagen, Inc., Hilden, Germany) with oligonucleotides containing the desired mutations. Substitutions of amino acids to alanine in cPDPN sequence were conducted by QuikChange lightning site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA). PCR fragments bearing the desired mutations were inserted into pCAG vector using In-Fusion PCR cloning kit (Clontech, Palo Alto, CA).

Flow cytometry

Transiently transfected CHO-K1 cells were detached by 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) (Nacalai Tesque, Inc.) and collected in RPMI 1640 medium. After washing with 0.1% bovine serum albumin /phosphate-buffered saline, the cells were incubated with anti-cPDPN antibody (PMab-52; 1 μg/mL) or control anti-MAP tag antibody (PMab-1; 1 μg/mL) for 30 minutes at 4°C followed by treatment with Alexa Fluor 488-conjugated antimouse IgG (1:1000; Cell Signaling Technology, Inc., Danvers, MA) and Oregon Green-conjugated antirat IgG (1:1000; Thermo Fisher Scientific, Inc.), respectively. Fluorescence data were collected using a Cell Analyzer EC800 (Sony Corp., Tokyo, Japan).

Western blot analysis

Whole cell lysates of the deletion and point mutants were collected, lysed, and then boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). The extracted proteins were electrophoresed using 5%–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Wako Pure Chemical Industries Ltd.) and transferred to polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt,

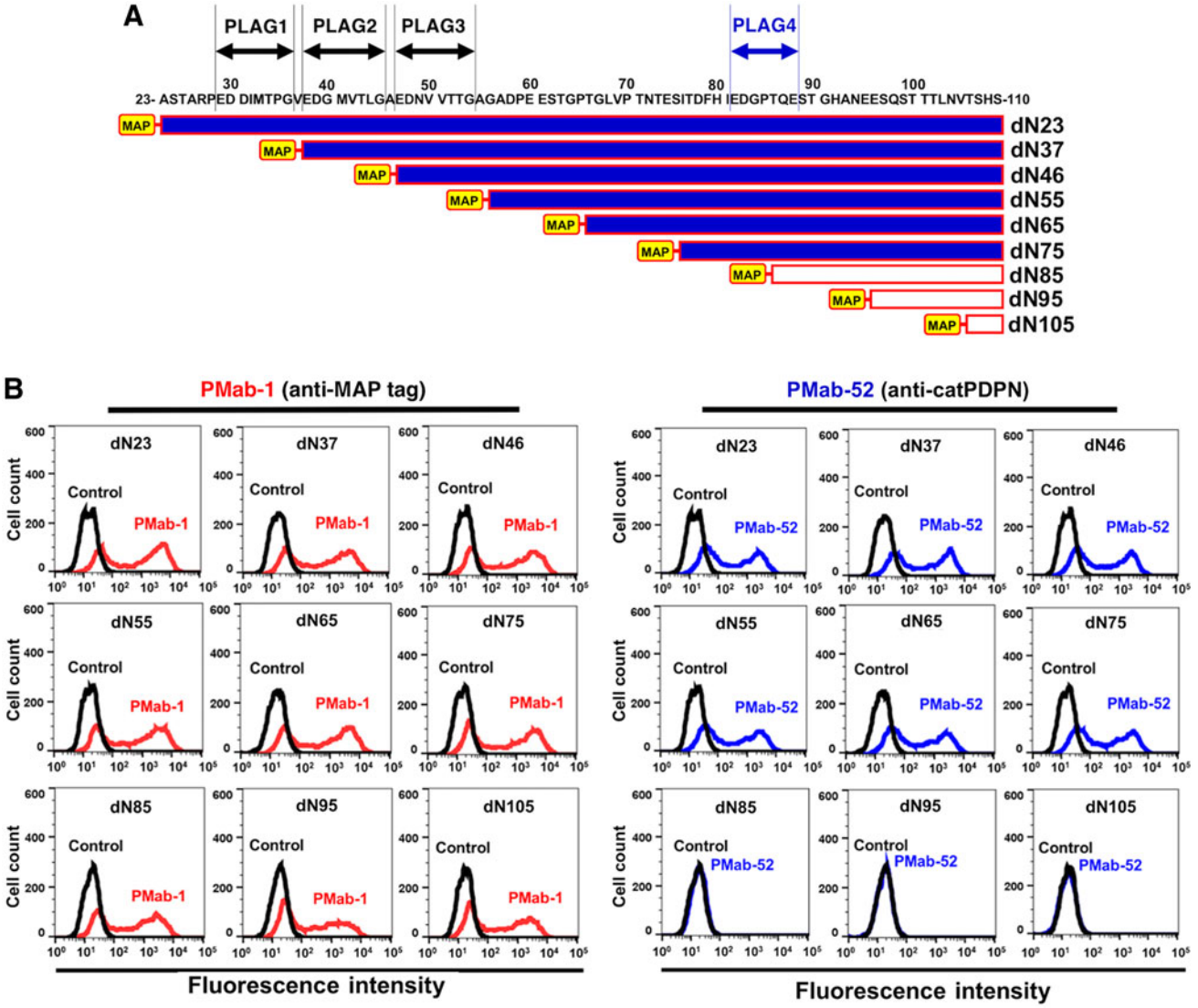

FIG. 1. Epitope mapping of PMab-52 using deletion mutants of cPDPN. **(A)** Illustration of nine cPDPN deletion mutants of dN23, dN37, dN46, dN55, dN65, dN75, dN85, dN95, and dN105. **(B)** Deletion mutants of cPDPN were analyzed using flow cytometry. Deletion mutants were expressed on CHO-K1 cells and were then incubated with anti-MAP tag PMab-1 (left panel, red line), PMab-52 (right panel, blue line), or buffer control (black line) for 30 minutes at 4°C, followed by treatment with corresponding secondary antibodies. CHO, Chinese hamster ovary; cPDPN, cat PDPN; PLAG4, platelet aggregation-stimulating domain 4.

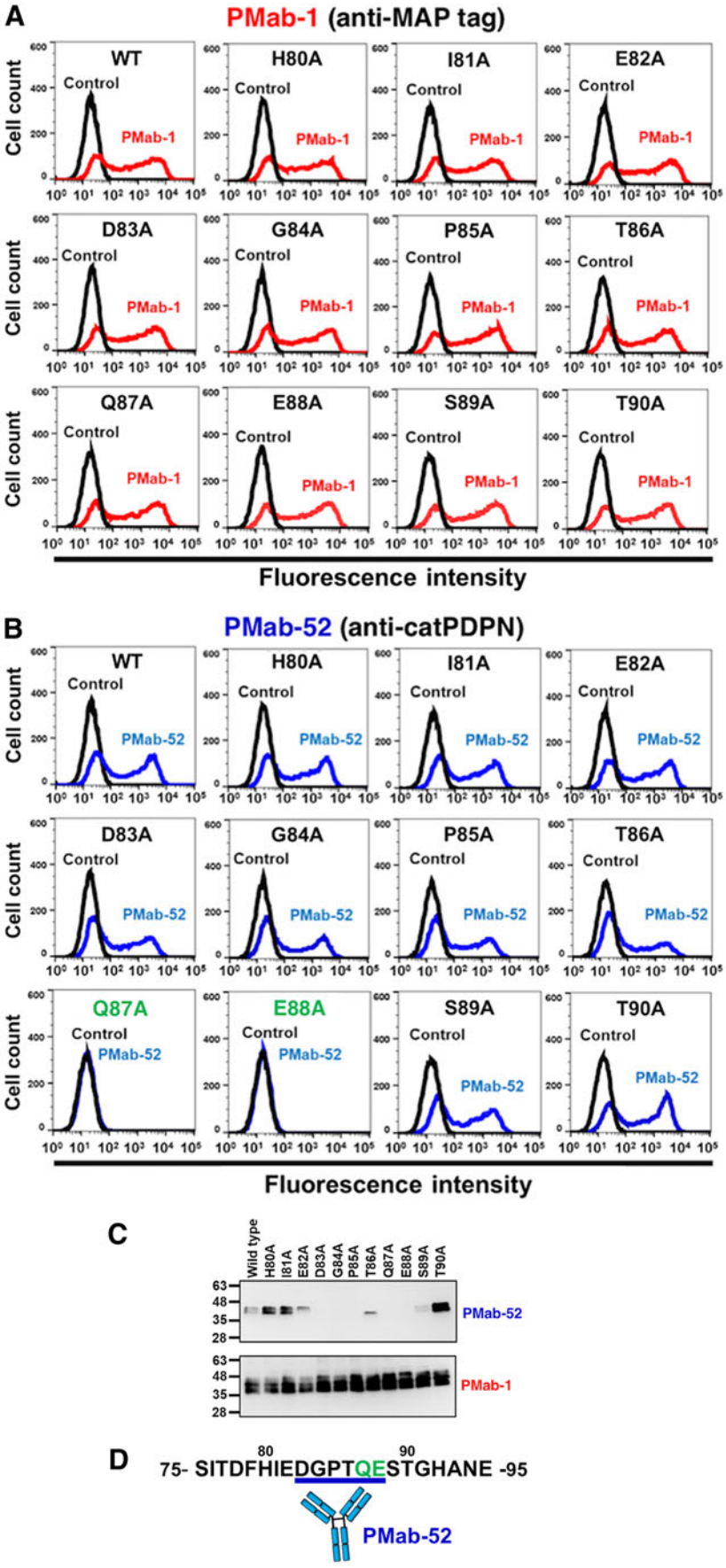

FIG. 2. Epitope mapping of PMab-52 using point mutants of cPDPN. **(A, B)** Flow cytometry. Transient point mutants expressing H80A, I81A, E82A, D83A, G84A, P85A, T86A, Q87A, E88A, S89A, and T90A of cPDPN reacted with PMab-1 **(A)** or PMab-52 **(B)** for 30 minutes at 4°C, followed by treatment with corresponding secondary antibodies. **(C)** The cell lysates from point mutants were collected for Western blotting with anti-cPDPN (PMab-52) antibody. The anti-MAP tag (PMab-1) antibody was used as a control. **(D)** Schematic illustration of the epitope recognized by PMab-52.

Germany). The membranes were blocked with 4% skim milk (Nacalai Tesque, Inc.) for 1 hour and then were incubated with 1 μg/mL of PMab-52 and 1 μg/mL of anti-MAP tag (PMab-1), then with peroxidase-conjugated antimouse and antirat IgG (1:1000; Agilent Technologies, Inc.), respectively. The proteins were finally detected using ImmunoStar LD (Wako Pure Chemical Industries Ltd.) with a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Results and Discussion

We have previously established a novel PMab-52 mAb, which can be efficiently utilized for the immunohistochemical detection of cPDPN in normal feline tissues, including kidney, lung, and rectum.[32] Furthermore, PMab-52 specifically recognized cPDPN expression in feline squamous cell carcinomas, including carcinomas of the mouth floor, skin, ear, and tongue.[33] Flow cytometry and Western blotting analyses also revealed that PMab-52 specifically detects only cPDPN, not PDPNs of other species such as humans, dogs, cattle, rabbits, and mice.[32] On the basis of these results, the epitope mapping of PMab-52 could be beneficial in uncovering the pathophysiological function of cPDPN in feline squamous cell carcinomas and cPDPN-related antibody-based therapy.

First, we constructed nine deletion mutants of cPDPN (Fig. 1A). Transient transfections of cPDPN-mutant clones were produced using CHO-K1 cells, including dN23 (corresponding to 23–163 amino acids [aa]); dN37 (corresponding to 37–163 aa); dN46 (corresponding to 46–163 aa); dN55 (corresponding to 55–163 aa); dN65 (corresponding to 65–163 aa); dN75 (corresponding to 75–163 aa); dN85 (corresponding to 85–163 aa); dN95 (corresponding to 95–163 aa); and dN105 (corresponding to 105–163 aa). All deletion mutants of cPDPN contain N-terminal MAP tags and were analyzed using flow cytometry for epitope mapping of PMab-52. PMab-1 (anti-MAP tag mAb) detected all deletion mutants of cPDPN, including dN23, dN37, dN46, dN55, dN65, dN75, dN85, dN95, and dN105 (Fig. 1B, left). On the contrary, PMab-52 lost the reaction with dN85, dN95, and dN105 (Fig. 1B, right). Further experiments revealed that PMab-52 could detect deletion mutants of dN80 of cPDPN (data not shown). These results imply that the epitope-binding region of PMab-52 is located between the 80th and 90th amino acids, which contain new identified platelet aggregation-stimulating domain 4 (PLAG4), which plays a crucial role in PDPN-mediated cancer metastasis.[34]

Next, we investigated the epitope-binding region of PMab-52 by producing 11 point mutants of cPDPN, including H80A, I81A, E82A, D83A, G84A, P85A, T86A, Q87A, E88A, S89A, and T90A. All these mutants can be recognized by PMab-1 (Fig. 2A). Remarkably, we observed that PMab-52 reacted with all these mutants, except with Q87A and E88A using flow cytometry (Fig. 2B).

To identify the epitope of cPDPN for PMab-52 binding, we also performed Western blotting using these point mutants. The results revealed that PMab-52 could not recognize the point mutants of D83A-P85A and Q87A-E88A (Fig. 2C), which are located in the PLAG4 domain ($_{82}$-EDGPTQE-$_{88}$). Both these results further confirmed that PMab-52 interacts with certain crucial amino acid residues (Asp83, Gly84, Pro85, Gln87, and Glu88) of the PLAG4 domain (Fig. 2D).

In conclusion, through this study, we characterized the crucial binding region of PLAG4 domain of cPDPN for PMab-52-specific binding. PMab-52 can be a useful tool in elucidating the pathophysiological function of cPDPN.

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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 Seiryo-machi
Aoba-ku
Sendai 980-8575
Japan

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

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