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Epitope Mapping of an Anti-Chinese/Golden Hamster Podoplanin Monoclonal Antibody

Nohara Goto,[1,*] Hiroyuki Suzuki,[1,*] Tomohiro Tanaka,[2] Teizo Asano,[2] Mika K. Kaneko,[2] and Yukinari Kato[1,2]

Chinese hamster (*Cricetulus griseus*) and golden hamster (*Mesocricetus auratus*) are important animal models of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections, which affect several organs, including respiratory tract, lung, and kidney. Podoplanin (PDPN) is a marker of lung type I alveolar cells, kidney podocytes, and lymphatic endothelial cells. The development of anti-PDPN monoclonal antibodies (mAbs) for these animals is essential to evaluate the pathogenesis by SARS-CoV-2 infections. Using the Cell-Based Immunization and Screening method, we previously developed an anti-Chinese hamster PDPN (ChamPDPN) mAb, PMab-281 (mouse IgG_3, kappa), and further changed its subclass into IgG_{2a} (281-mG_{2a}-f), both of which can recognize not only ChamPDPN but also golden hamster PDPN (GhamPDPN) by flow cytometry and immunohistochemistry. In this study, we examined the critical epitope of 281-mG_{2a}-f, using enzyme-linked immunosorbent assay (ELISA) with synthesized peptides. First, we performed ELISA with peptides derived from ChamPDPN and GhamPDPN extracellular domain, and found that 281-mG_{2a}-f reacted with the peptides, which commonly possess the KIPFEELxT sequence. Next, we analyzed the reaction with the alanine-substituted mutants, and revealed that 281-mG_{2a}-f did not recognize the alanine-substituted peptides of I75A, F77A, and E79A of ChamPDPN. Furthermore, these peptides could not inhibit the recognition of 281-mG_{2a}-f to ChamPDPN-expressing cells by flow cytometry. The results indicate that the binding epitope of 281-mG_{2a}-f includes Ile75, Phe77, and Glu79 of ChamPDPN, which are shared with GhamPDPN.

Keywords: hamster 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 tumor progression[1] as well as normal development including lung,[2] kidney,[3] and lymphatic vessels.[4] The N-terminal extracellular domain has a repeat sequence named platelet aggregation-stimulating (PLAG)1 to PLAG3 domains[5] that promote platelet aggregation through interaction with a platelet receptor, C-type lectin-like receptor 2 (CLEC-2).[6,7] Furthermore, several PLAG-like domains (PLDs, one of which is named PLAG4 domain) with similar sequences, were identified.[8]

Furthermore, PDPN regulates the signal transduction through its cytoplasmic tail, which is involved in cell proliferation, migration, invasion, epithelial-to-mesenchymal transition, and stemness.[9] PDPN expression is also elevated in tumor stroma including cancer-associated fibroblasts (CAFs)[10] and lymphocytes.[11] CAFs remodel the extracellular matrix and the play a critical role in the formation of immunosuppressive tumor microenvironment.[12,13]

PDPN is also important as a marker of lung type I alveolar cells, kidney podocytes, and lymphatic endothelial cells.[1] We have developed anti-PDPN monoclonal antibodies (mAbs) against 17 species,[14–30] which are useful for flow cytometry and immunohistochemistry. These mAbs are expected to contribute not only to the research of each animal but also to pathogenic diagnosis.

Using the Cell-Based Immunization and Screening (CBIS) method,[14,15,31–45] we recently developed anti-PDPN mAbs against Chinese hamster (Cham)/golden hamster (Gham)[46] and ferret,[47] which are small animal models of severe acute

Departments of [1]Molecular Pharmacology and [2]Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
*These authors contributed equally to this study.

respiratory syndrome coronavirus 2 (SARS-CoV-2) infections.[48,49] These mAbs will contribute to the morphological alterations and evaluation of the pathogenesis of SARS-CoV-2-infected lung type I alveolar cells and kidney podocytes. In this study, we performed the epitope mapping using enzyme-linked immunosorbent assay (ELISA) to clarify further characteristics of an anti-Cham/GhamPDPN mAb, 281-mG_{2a}-f.

Materials and Methods

Peptides

ChamPDPN (accession no.: AB205160) and GhamPDPN (accession no.: XM_021233536) peptides (Table 1) and 20 alanine-substituted peptides (Table 2) were synthesized by utilizing PEPScreen (Sigma-Aldrich Corp., St. Louis, MO).

ELISA

Synthesized ChamPDPN and GhamPDPN peptides (Tables 1 and 2) 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 281-mG_{2a}-f, 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).

Table 1. Epitope Mapping of 281-mG_{2A}-f Using Deletion Mutants

Peptides	*Sequences*	*281-mG_{2a}-f*
ChamPDPN		
23–42	GAIGRLEDDIVTPGARDGMV	–
33–52	VTPGARDGMVTPGLEDRIGT	–
43–62	TPGLEDRIGTTGATEVLNES	–
53–72	TGATEVLNESTGKAPLVPTH	–
63–82	TGKAPLVPTHTKIPFEELPT	+++
73–92	TKIPFEELPTPGISDHDGEE	+++
83–102	PGISDHDGEEHTSTTTVRMV	–
93–112	HTSTTTVRMVTSHSADKETS	–
103–122	TSHSADKETSHPNRDNTADE	–
113–132	HPNRDNTADETQTTDKRDGL	–
123–135	TQTTDKRDGLAVV	–
GhamPDPN		
48–67	ALLKGLEDDIVTPGARDGMV	–
58–77	VTPGARDGMVTPGLEDRTTT	–
68–87	TPGLEDRTTTTGGLNEPTGK	–
78–97	TGGLNEPTGKAPLVPTHAKI	–
88–107	APLVPTHAKIPFEELSTPGV	+++
98–117	PFEELSTPGVSDHDDKEHKS	–
108–127	SDHDDKEHKSTTTVRMVTSH	–
118–137	TTTVRMVTSHSSDKETSHPN	–
128–147	SSDKETSHPNIDNTADETQT	–
138–157	IDNTADETQTTDKRDGLAVV	–
148–157	TDKRDGLAVV	–

+++, OD655 ≧ 0.3; –, OD655 < 0.1.
ChamPDPN, Chinese hamster PDPN; GhamPDPN, golden hamster PDPN; PDPN, podoplanin.

Table 2. Identification of the 281-mG_{2A}-f Epitope Using Alanine-Substituted Chinese Hamster Podoplanin Peptides

Peptides	*Sequences*	*281-mG_{2a}-f*
73–92	TKIPFEELPTPGISDHDGEE	+++
T73A	AKIPFEELPTPGISDHDGEE	+++
K74A	TAIPFEELPTPGISDHDGEE	+++
I75A	TKAPFEELPTPGISDHDGEE	–
P76A	TKIAFEELPTPGISDHDGEE	+++
F77A	TKIPAEELPTPGISDHDGEE	–
E78A	TKIPFAELPTPGISDHDGEE	+++
E79A	TKIPFEALPTPGISDHDGEE	–
L80A	TKIPFEEAPTPGISDHDGEE	+++
P81A	TKIPFEELATPGISDHDGEE	+++
T82A	TKIPFEELPAPGISDHDGEE	+++
P83A	TKIPFEELPTAGISDHDGEE	+++
G84A	TKIPFEELPTPAISDHDGEE	+++
I85A	TKIPFEELPTPGASDHDGEE	+++
S86A	TKIPFEELPTPGIADHDGEE	+++
D87A	TKIPFEELPTPGISAHDGEE	+++
H88A	TKIPFEELPTPGISDADGEE	+++
D89A	TKIPFEELPTPGISDHAGEE	+++
G90A	TKIPFEELPTPGISDHDAEE	+++
E91A	TKIPFEELPTPGISDHDGAE	+++
E92A	TKIPFEELPTPGISDHDGEA	+++

+++, OD655 ≧ 0.3; –, OD655 < 0.1.

Flow cytometry

2×RIEDL-ChamPDPN-overexpressed Chinese hamster ovary-K1 (CHO/ChamPDPN)[46] was harvested after a brief exposure to 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.) and washed with 0.1% BSA (Nacalai Tesque, Inc.) in PBS (Nacalai Tesque, Inc.). The 281-mG_{2a}-f (0.01 μg/mL) was incubated with each peptide (10 μg/mL) for 30 minutes at 4°C. CHO/ChamPDPN cells were treated with 281-mG_{2a}-f + each peptide, and further treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000). Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Biotechnology Corp., Tokyo, Japan).

Results

Epitope mapping of 281-mG_{2a}-f using deletion mutants

We previously established an anti-ChamPDPN mAb (PMab-281, mouse IgG_3, kappa) by the CBIS method.[46] The subclass of PMab-281 was converted from IgG_3 to IgG_{2a} because the mouse IgG_3 subclass is easy to aggregate. In addition, a defucosylated anti-ChamPDPN mAb (281-mG_{2a}-f) was produced using BINDS-09 cells (FUT8-deficient ExpiCHO-S cells[50,51]). We found that 281-mG_{2a}-f could recognize both ChamPDPN and GhamPDPN by flow cytometry and immunohistochemistry.[46] To reveal the binding epitope of 281-mG_{2a}-f, we synthesized 22 peptides (Table 1), which consist of 20 amino acids (aa) of extracellular domain of ChamPDPN and GhamPDPN, and performed ELISA. As shown in Figure 1A, 281-mG_{2a}-f recognized both the 63–82 aa (TGKAPLVPTHTKIPFEELPT) and the 73–92 aa

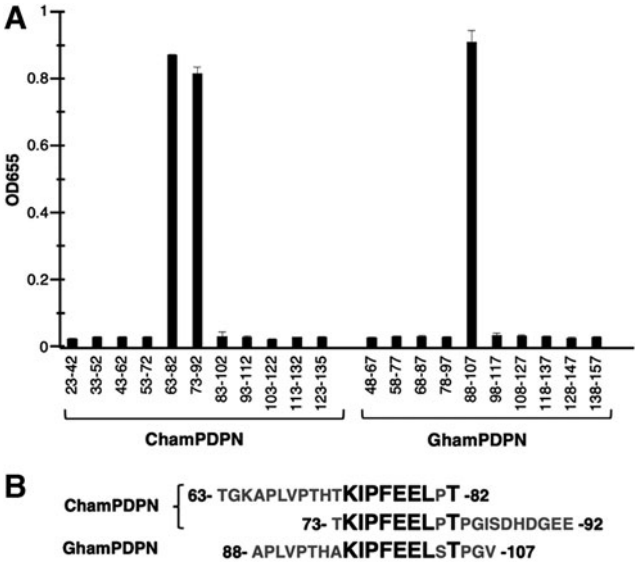

FIG. 1. Determination of the 281-mG_{2a}-f epitope for ChamPDPN and GhamPDPN by ELISA using deletion mutants. **(A)** Synthesized peptides of ChamPDPN and GhamPDPN were immobilized on immunoplates. The plates were incubated with 281-mG_{2a}-f (1 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Sequence alignment of reacted peptides. ChamPDPN, Chinese hamster PDPN; ELISA, enzyme-linked immunosorbent assay; GhamPDPN, golden hamster PDPN; PDPN, podoplanin.

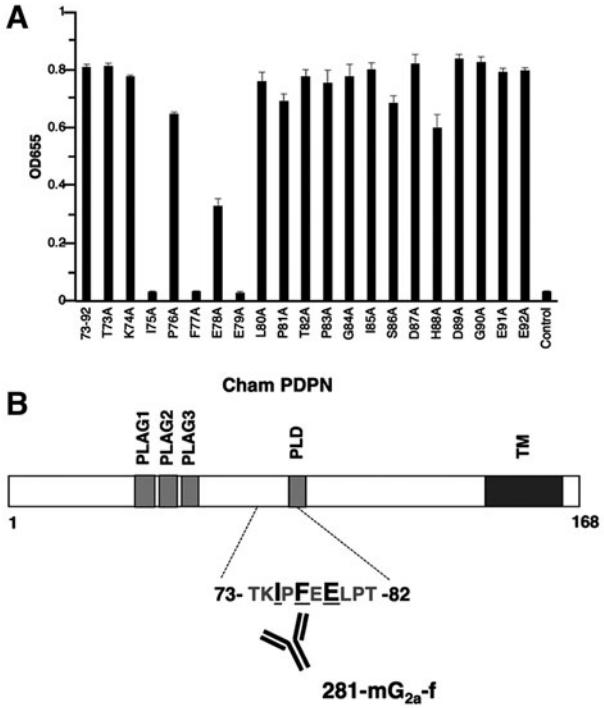

FIG. 2. Determination of the 281-mG_{2a}-f epitope of ChamPDPN by ELISA using alanine-substituted PDPN peptides. **(A)** The alanine-substituted ChamPDPN peptides were immobilized on immunoplates. The plates were incubated with 281-mG_{2a}-f (1 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of ChamPDPN and the 281-mG_{2a}-f epitope. The 281-mG_{2a}-f epitope involves Ile75, Phe77, and Glu79 of ChamPDPN.

(TKIPFEELPTPGISDHDGEE) sequences of ChamPDPN. Furthermore, 281-mG_{2a}-f recognized the 88–107 aa (APLVPTHAKIPFEELSTPGV) sequence of GhamPDPN. Compared with the sequences, the epitope of 281-mG_{2a}-f was suggested to exist in the 74–82 aa (KIPFEELPT) of ChamPDPN (Fig. 1B).

Epitope mapping of 281-mG_{2a}-f using alanine-substituted PDPN peptides

Then, we synthesized 20 alanine-substituted peptides derived from the 73–92 aa peptide of ChamPDPN (Table 2). The 281-mG_{2a}-f exhibited reaction with T73A, K74A, P76A, E78A, L80A, P81A, T82A, P83A, G84A, I85A, S86A, D87A, H88A, D89A, G90A, E91A, E92A, and wild-type (WT, 73–92 aa) (Fig. 2A). In contrast, 281-mG_{2a}-f did not react with I75A, F77A, and E79A (Fig. 2A), indicating that Ile75, Phe77, and Glu79, which are shared with GhamPDPN, are included in the critical epitope of 281-mG_{2a}-f. The results are summarized in Figure 2B.

Flow cytometry using 281-mG_{2a}-f with alanine-substituted PDPN peptide

We performed a blocking assay using flow cytometry. As shown in Figure 3, 281-mG_{2a}-f reacted with the CHO/ChamPDPN cells. This reaction was almost completely neutralized by WT and L80A, partially inhibited by K74A and P76A, and slightly inhibited by E78A. In contrast, I75A, F77A, and E79A did not block the reaction of 281-mG_{2a}-f with CHO/ChamPDPN. These results confirm that Ile75, Phe77, and Glu79 of ChamPDPN are critical for 281-mG_{2a}-f detection.

Discussion

PDPN possesses three tandem repeats of the ''EDxxVTPG'' sequences, which were defined as PLAG1, PLAG2, and PLAG3 domains in the N-terminus.[5] Furthermore, there are several PLDs of the ''E(D/E)xx(T/S)xx'' sequences in the central part of PDPN.[8] In this study, we determined the critical epitope of 281-mG_{2a}-f as Ile75, Phe77, and Glu79 of ChamPDPN (Fig. 2B). Glu79 is included in the first PLD (aa 78–82).

Since PLDs are reportedly important for the PDPN–CLEC-2 interaction and induction of platelet aggregation,[8] further studies are needed to investigate the role of the first PLD for platelet aggregation and the neutralizing activity of 281-mG_{2a}-f. The *O*-glycosylation of Thr in the PLAG3 or PLD has been reported to be essential for PDPN-induced platelet aggregation.[8,52] However, Ser/Thr residues are not included in 281-mG_{2a}-f epitope, indicating that 281-mG_{2a}-f was not categorized into GpMabs.[53]

We have not examined the crossreactivity of 281-mG_{2a}-f with other species excluding Gham. Therefore, we searched the conservation of ''IPFEE'' sequence to other species PDPN using standard protein BLAST (the Basic Local

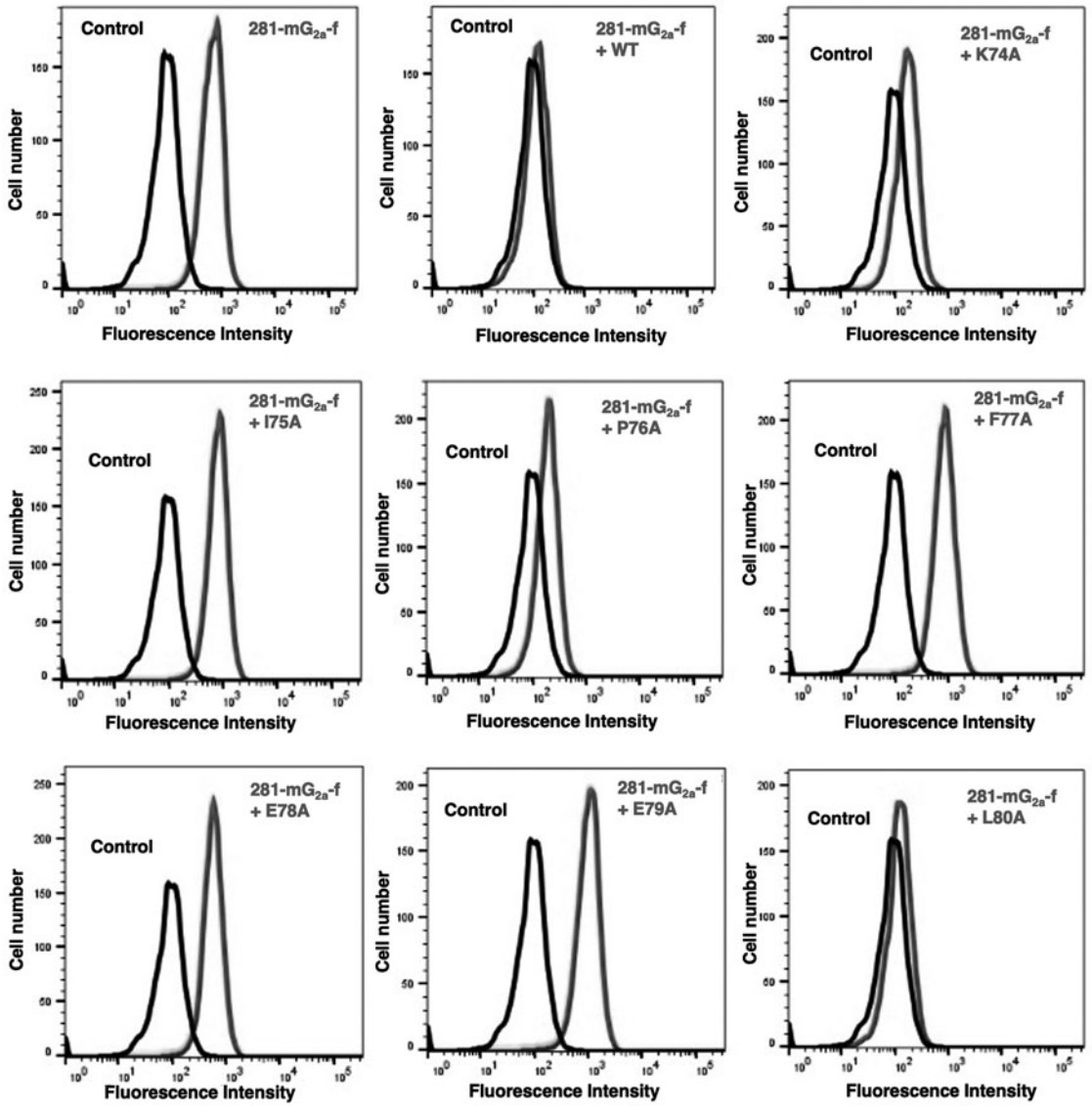

FIG. 3. Flow cytometry using 281-mG_{2a}-f and peptides of ChamPDPN. The 281-mG_{2a}-f (0.01 μg/mL), 281-mG_{2a}-f (0.01 μg/mL) plus the alanine-substituted peptides (10 μg/mL), or control (blocking buffer) were reacted with CHO/ChamPDPN cells for 30 minutes at 4°C, followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. CHO, Chinese hamster ovary-K1.

Alignment Search Tool, NCBI). Only creeping vole (*Microtus oregoni*) possesses similar ''IPFED'' sequence, suggesting the crossreactivity by 281-mG_{2a}-f.

In animal models of SARS-CoV-2, Ghams exhibit similar pathogenesis and transmissibility found in humans with SARS-CoV-2 infections.[54] The disease severity is known to be typically lower in pediatric patients than in adults, particularly the elderly patients.[55] Angiotensin-converting enzyme 2 (ACE2) serves as the entry receptor for SARS-CoV-2.[56] Age-dependent upregulation of ACE2 in PDPN-positive lung type I alveolar cells was reported in mouse and human.[57] The 281-mG_{2a}-f will contribute to the analysis to detect PDPN-positive lung type I alveolar cells in Gham and could provide an important information of the age-related correlation of disease severity in the animal model.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Yukinari Kato
Department of Molecular Pharmacology
Tohoku University Graduate School of Medicine
2-1, Seiryo-machi, Aoba-ku
Sendai 980-8575
Japan

E-mail: yukinarikato@med.tohoku.ac.jp

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