FEBS Letters 581 (2007) 331–336

Functional glycosylation of human podoplanin: Glycan structure of platelet aggregation-inducing factor

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Received 4 December 2006; revised 20 December 2006; accepted 21 December 2006

Available online 5 January 2007

Edited by Judit Ovádi

Abstract **Podoplanin (Aggrus) is a mucin-type sialoglycoprotein that plays a key role in tumor cell-induced platelet aggregation. Podoplanin possesses a platelet aggregation-stimulating (PLAG) domain, and Thr52 in the PLAG domain of human podoplanin is important for its activity. Endogenous or recombinant human podoplanin were purified, and total glycosylation profiles were surveyed by lectin microarray. Analyses of glycopeptides produced by Edman degradation and mass spectrometry revealed that the disialyl-corel (NeuAcα2-3Galβl-3(NeuAcα2-6)GalNAcαl-*O*-Thr) structure was primarily attached to a glycosylation site at residue Thr52. Sialic acid-deficient podoplanin recovered its activity after additional sialylation. These results indicated that the sialylated Corel at Thr52 is critical for podoplanin-induced platelet aggregation.**

Keywords: Podoplanin; Platelet aggregation; Disialyl-corel, Disialyl-T

1. Introduction

It is well known that tumor metastasis is associated with platelet-aggregating activity possessed by human and other mammalian cancer cells [1]. A previous study has clarified that podoplanin, membranous 44 kDa and 36 kDa sialoglycoproteins in cancer cells of mice and humans, respectively, aggregated platelets with no relation to plasma components [2,3]. Podoplanin is known to be a lymphatic specific marker [4], and its expression has been reported in many tumor cells [4–11]. Podoplanin belongs to a type-I transmembrane sialomucin-like glycoprotein that consisted of an extracellular domain with abundant Ser and Thr residues as potential *O*-glycosylation sites, a single transmembrane portion, and a short cytoplasmic tail with putative sites for protein kinase C and cAMP phosphorylation [2,12].

We previously showed that the segment of EDxxVTPG in the extracellular domain, designated as platelet aggregation-stimulating (PLAG) domain, was critical for the activity of podoplanin/Aggrus [2]. In particular, this motif, which is highly conserved among species, is tandemly triplicated [13]. In the study of targeted mutagenesis to podoplanin molecules, we obtained evidence that Thr residues of PLAG domain play an important role for platelet aggregation [2,13]. Furthermore, unique characteristics of Chinese hamster ovary (CHO) mutant cell lines, *N*-glycan-deficient Lecl, CMP-sialic acid transporter-deficient Lec2, and UDP-galactose transporter-deficient Lec8, revealed that sialylated *O*-glycan is critical for platelet aggregation-inducing activity [14].

In this study, we purified human podoplanin from a glioblastoma cell line LN319 cells and podoplanin-transfected CHO cells. Using a lectin microarray, Edman degradation, and matrix-assisted laser desorption/ionization time of-flight mass spectrometry (MALDI-TOF-MS), it was shown that podoplanin possesses a disialyl-corel structure at Thr52 in the PLAG domain, and this structure is critical for its platelet-aggregating activity.

2. Materials and methods

2.1. Production and purification of podoplanin

We previously established FLAG-tagged human podoplanin-transfected CHO and Lec2 cell lines [14]. A glioblastoma cell line LN319, which expresses endogenous podoplanin, was donated by Dr. Webster K Cavenee [15]. Cells were lysed with 0.25% Triton X-100 in PBS. Using anti-FLAG antibody-conjugated agarose gel (M2, Sigma), podoplanin was purified from podoplanin-transfected CHO and Lec2 cells, and eluted using 100 μg/ml FLAG peptide (Sigma). Endogenous podoplanin was purified from LN319 using anti-human podoplanin antibody (NZ-1) and eluted by hpp38–51 peptide (EGGVAMPGAEDDVV) [15].

2.2. Platelet aggregation assay

The platelet aggregation assay was described previously [15]. Briefly, platelet aggregation was measured by WBA Carna (M.C. Medical)

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Abbreviations: PLAG, platelet-aggregation-stimulating; CHO, Chinese hamster ovary; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of-flight mass spectrometry; QIT, quadrupole ion trap; HPLC, high performance liquid chromatography

0014-5793/$32.00
doi:10.1016/j.febslet.2006.12.044

with the screen filtration pressure method [16]. Two hundred microliters each of heparinized mouse whole blood samples was pre-incubated for 2 min, followed by the addition of 12 μl each of purified protein. Five minutes later, samples were sucked to detect aggregation pressure. The final platelet aggregation pressure of each reaction tube was determined at the pressure rate (%) of a pressure sensor connected to the syringe.

2.3. Lectin microarray

The lectin microarray was basically performed as described by Kuno et al. [17].

To generate glycan profiles for podoplanin, we customized a procedure using the NZ-1 antibody [15]. Interaction of podoplanin with the lectin immobilized on the glass slide was detected using biotinylated NZ-l-Cy3-streptoavidin method to profile glycans of podoplanin. Briefly, purified podoplanin was diluted to 60 μl with 1% Trition X-100 in PBS (PBSTx) and applied to the lectin array containing triplicate spots of 43 lectin into each of eight-divided incubation baths on the glass slide (refer to [15]). After incubation at 20 °C for 12 h, the reaction solution was discarded. The glass slide was washed three times with PBSTx; 60 μl of biotinylated NZ-1 antibody (0.17 μg/ml) in PBS was applied to the array and then incubated at 20 °C for 3 h. After washing three times with PBSTx, Cy3-labeled streptavidin (GE Healthcare, UK) was added to the array and then incubated at 20 °C for 30 min. The glass slide was rinsed with PBSTx and scanned using an evanescent-field fluorescence scanner (GTMASScan III; Nippon Laser & Electronics Lab, Nagoya, Japan). Lectin binding was measured by fluorescent intensity.

2.4. Mass spectrometry

Beta-Elimination [18] and permethylation [19] were performed as described previously. Desialylation was performed at 80 °C for 3 h in 2 M acetic acid. MS measurements were carried out using a positive reflectron mode for glycans, and negative reflectron mode for glycopeptide with a MALDI-TOF mass spectrometer (Reflex IV; Bruker-Daltonik, Germany). All collision-induced dissociation spectra were acquired with a MALDI-QIT (quadrupole ion trap)-TOF MS spectrometer (AXIMA-QIT; Shimadzu, Kyoto, Japan). For each sample preparation 2,5-DHB was used as the matrix. MS^n fingerprint matching was performed as described previously [20].

2.5. Endoproteinase digest, peptide separations, and protein sequencing

Using purified and concentrated hPod/LN319 and hPod/CHO, endoproteinase Asp-N digestion was performed in 50 mM Tris–HCl (pH 8.0) at 37 °C for 8 h. The digests were separated by reverse phase chromatography with a CAPCELLPAC C18 UG120 column (SHISEIDO) using high performance liquid chromatography (HPLC, Shimadzu). A linear gradient (0–50%) of acetonitrile in 0.1% trifluoroacetic acid was performed at 40 °C. The glycosylation site on podoplanin was determined by Edman microsequencing (PPSQ-23A, Shimazu), since phenylthiohydantoin derivatives of glycosylated-Thr were not detected.

2.6. Addition of sialic acid

Sialylation experiments were described previously [21,22]. For the preparation of hPod/Lec2 with the sialyl Corel (NueAcα2-3Galβl-3GalNAcαl-) residue, 25 mM HEPES buffer (pH 7.0) containing 30 μl of acceptor substrate (1.0 mg/ml), 10 mM $MnCl_2$ and an appropriate concentration of CMP-NeuAc was used. Ninety microliters of purified ST3GalI enzyme was added to the reaction mixture and incubated at 37 °C for 20 h. For the preparation of hPod/Lec2 with the disialyl-corel (NueAcα2-3Galβl-3(NeuAcα2-6)GalNAcαl-) residue, aliquots of supernatants of the former reaction solution were used as the acceptor substrate for the next sialylation. An appropriate concentration of CMP-NeuAc and the purified ST6GalNAcI enzyme were added to 30 μl of supernatants and incubated at 37 °C for 20 h. Control hPod/Lec2 sample was also treated in the same experimental condition except for the addition of ST3GalI or ST6GalNAcI.

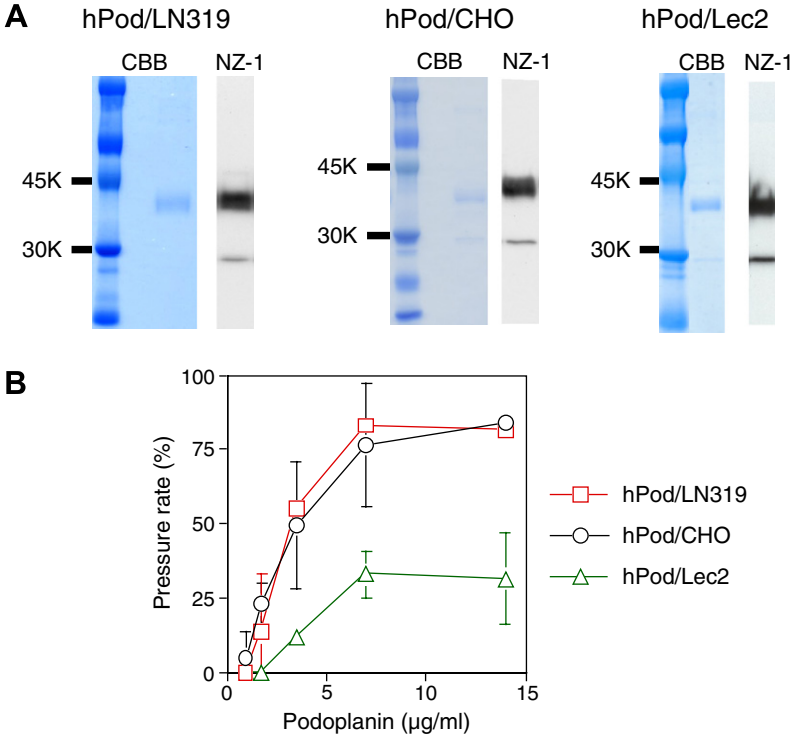

Fig. 1. Purification and platelet aggregation assay for endogenous and recombinant podoplanin. (A) Affinity-purified human podoplanin from LN319, podoplanin-transfected CHO, and podoplanin-transfected Lec2 cell lines were eluted using 100 μg/ml hpp38–51 peptide or FLAG peptide. Purified proteins were electrophoresed, CBB stained, and immunoblotted with NZ-1 antibody. (B) Purified and concentrated podoplanins from LN319, podoplanin-transfected CHO, and podoplanin-transfected Lec2 induced platelet aggregation. Platelet aggregation was measured using WBA Carna with the screen filtration pressure method.

3. Results and discussion

3.1. Purification of human podoplanin and platelet aggregation assay

Purifications of endogenous or recombinant podoplanin from LN319, podoplanin-transfected CHO, and podoplanin-transfected Lec2 were achieved by a one-step procedure using immunoaffinity chromatography utilizing a monoclonal antibody (NZ-1 [15] or M2). Purified podoplanin proteins (hPod/LN319, hPod/CHO, and hPod/Lec2) were confirmed to be more than 95% pure based on CBB staining (Fig. 1A). All purified podoplanins induced platelet aggregation in a dose-dependent manner (Fig. 1B). In our previous study, we indicated that human podoplanin possessed the ability to induce aggregation of human and mouse platelets [2]. Furthermore, platelet aggregation induced by human podoplanin using mouse platelet was reported to be stronger than one using human platelet. Therefore, we used mouse platelet in this whole study. We have investigated the platelet aggregation induced by podoplanin using both a turbidimetric method with platelet-rich plasma (PRP) and a filtration pressure method with whole blood, and obtained the compatible results using both methods (data not shown). Lec2 cells are CMP-sialic acid transporter-deficient in which both glycoproteins and glycolipids lack 90% of sialic acids (SA) [23]. hPod/Lec2 possessed a trace amount of SA on the purified protein (Fig. 2). Therefore, hPod/Lec2 protein could induce platelet aggregation when it was purified and concentrated.

3.2. Total glycan profiling of podoplanin of CHO and LN319 using lectin microarray

We investigated the total glycan profile of hPod/LN319, hPod/CHO, and hPod/Lec2 using a lectin microarray with the detection method by biotinylated NZ-1 antibody and Cy3-labeled streptavidin [15]. Lectin binding was measured by fluorescent intensity. As shown in Fig. 2, both hPod/LN319 and hPod/CHO reacted strongly with corel ± sialic acid binders (ABA, Jacalin, ACA, and MPA) and sialo-mucin binders (MAH and WGA), but did not react with corel binders (BPL and PNA). These results indicated that podoplanin on LN319 or podoplanin-transfected CHO possesses a disialyl- and/or monosialyl-corel structure. Predictably, hPod/Lec2 reacted intensely to corel binders (BPL and PNA). Residual sialic acid of hPod/Lec2 was detected by WGA. In particular, Jacalin and MPA reactivity of hPod/CHO differed from that of hPod/LN319. Jacalin did not bind to disialyl-corel structure [24]. Accordingly, these results suggested that hPod/LN319 contains more disialyl-corel compared to hPod/CHO.

3.3. Glycan analysis of podoplanin of CHO and LN319 using mass spectrometry

Next, the total glycans of hPod/LN319 and hPod/CHO were analyzed by mass spectrometry. Glycans were released from hPods by reductive β-elimination [18]. The resulting alditols were permethylated [19] and analyzed with MALDI-TOF and MALDI-QIT-TOF mass spectrometers. MS spectra revealed that hPod/CHO bears nearly equal amounts of $(NeuAc)_2$-Hex-HexNAc-ol (*m/z* 1257) and NeuAc-Hex-HexNAc-ol (*m/z* 895), whereas hPod/LN319 bears mainly $(NeuAc)_2$-Hex-HexNAc-ol (Fig. 3A). In the MS/MS spectrum of *m/z* 1257, the signals of *m/z* 620 and 659, which could be assigned as $[permethylated\ NeuAc\text{-}Hex + Na]^+$ and $[permethylated\ NeuAc\text{-}HexNAc\text{-}ol + Na]^+$, were observed (Fig. 3B). All of these results indicated that the glycans found on podoplanin, in LN319 and podoplanin-transfected CHO, are mixture of monosialyl- and disialyl-corel structures.

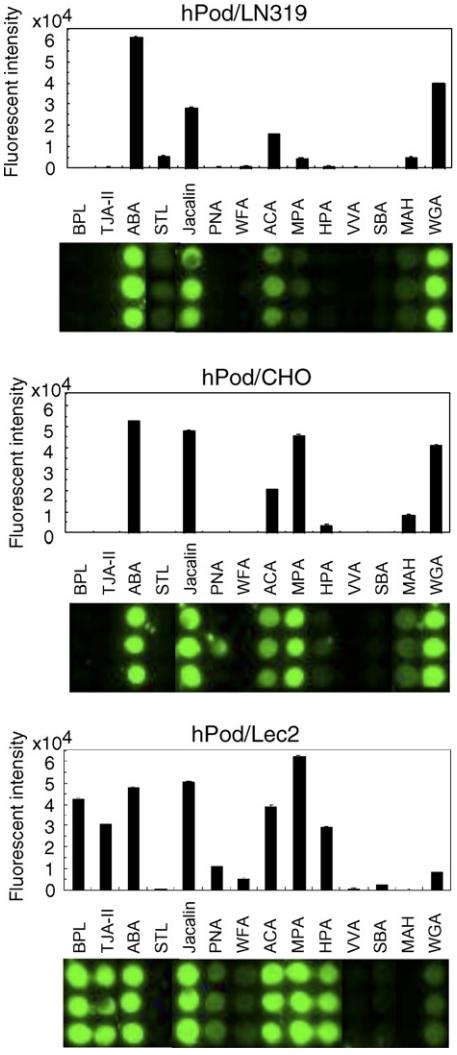

Fig. 2. Determination of total glycan structure for endogenous and recombinant podoplanin. Glycan profiles of purified podoplanins from LN319, podoplanin-transfected CHO, and podoplanin-transfected Lec2 cells using lectin microarray. The fluorescent intensities of each lectin on lectin array are indicated with bars. Lectin binding specificities were as follows: BPL; Galβ1-3GlaNAc, TJA-II; β-GalNAc, ABA: Galβ1-3GlaNAcα-Thr/Ser (T) and sialyl-T, STL; (GlcNAc)*n*, Jacalin; Galβ1-3GlaNAcα-Thr/Ser and GlaNAcα-Thr/Ser, PNA Galβ1-3GalNAcα-Thr/Ser, WFA; terminal GalNAc, ACA; Galβ1-3GalNAcα-Thr/Ser, MPA; Galβ1-3GlaNAcα-Thr/Ser and GlaNAcα-Thr/Ser, HPA; terminal GalNAc,VVA; terminal GalNAc and GlaNAcα-Thr/Ser, SBA; terminal GalNAc, MAH; Siaα2-3Galβ1–3 (Siaα2–6)GalNAcα-R, WGA; (GlcNAc)*n* and multivalent Sia.

3.4. Glycosylation site of functional podoplanin

Mutation of threonine residues in the PLAG domain (52th threonine (Thr52) of human podoplanin) abolished the platelet aggregation-inducing abilities [2]. Furthermore, Thr52 was predicted as a *O*-glycosylated site [13]. In this study, glycopeptides from hPod/LN319 and hPod/CHO digested by Asp-N were fractionated by HPLC, followed by peptide sequencing,

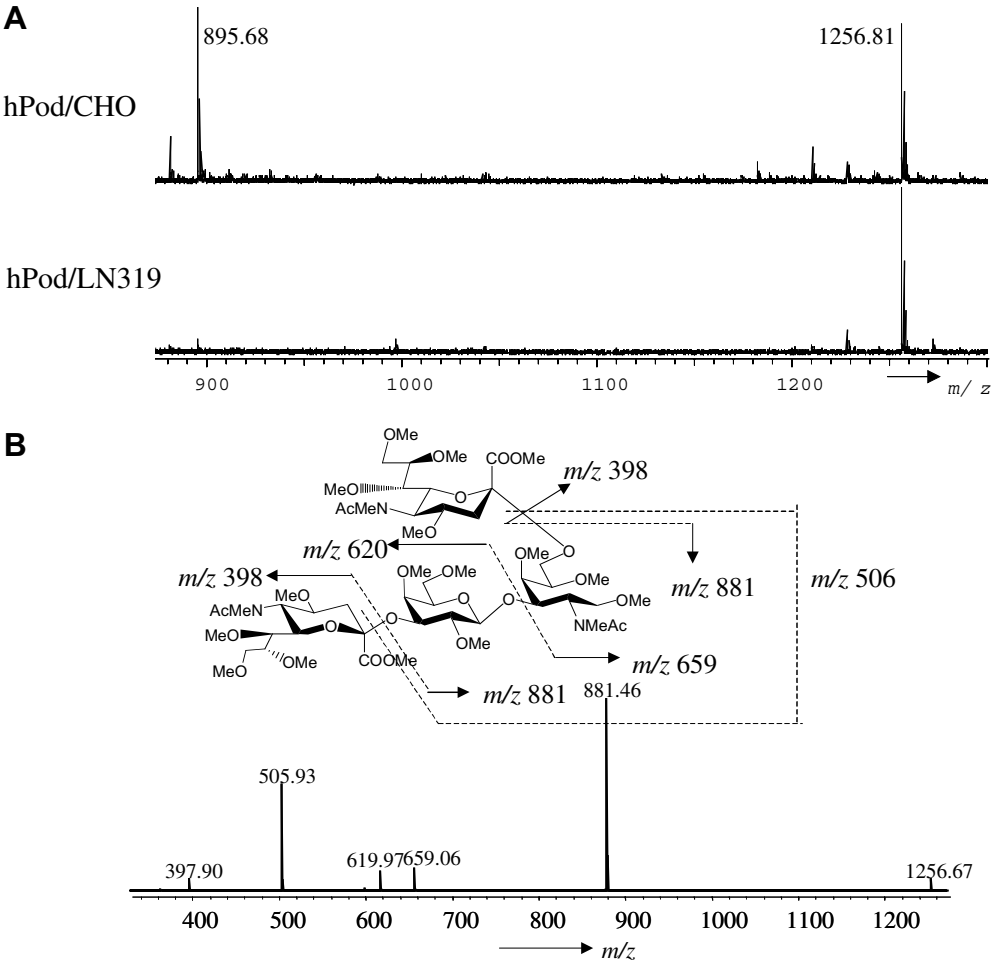

Fig. 3. The glycan structure of podoplanin analyzed by mass spectrometry. (A) MS spectra of permethylated alditols released from purified hPods/LN319 and hPods/CHO. *M/z* 895.68: NeuAc-Hex-HexNAc-ol, 1256.81: $(NeuAc)_2$-Hex-HexNAc-ol. (B) MS/MS spectrum of *m/z* 1257. As shown in the inset, *m/z* 620 and 659 could be assigned as [permethylated NeuAc-Hex + Na]$^+$ and [permetylated NeuAc-HexNac-ol+Na]$^+$.

and a glycopeptide consisting of Ala23–Glu57 was obtained, in which four potential Asp-N digestion sites were missed. The sequencing gap was observed at only Thr52 during Edman degradation from Ala23 to Glu57 (Fig. 4). Other possible *O*-glycosylation sites, such as Thr32, Thr34, and Thr55 (not shown for others) gave the correct sequences. Therefore, only Thr52 was *O*-glycosylated in this peptide. Furthermore, we confirmed using undigested hPod/LN319 and hPod/CHO proteins that Ala23 was the N-terminal amino acid of both endogenous and recombinant podoplanin (data not shown).

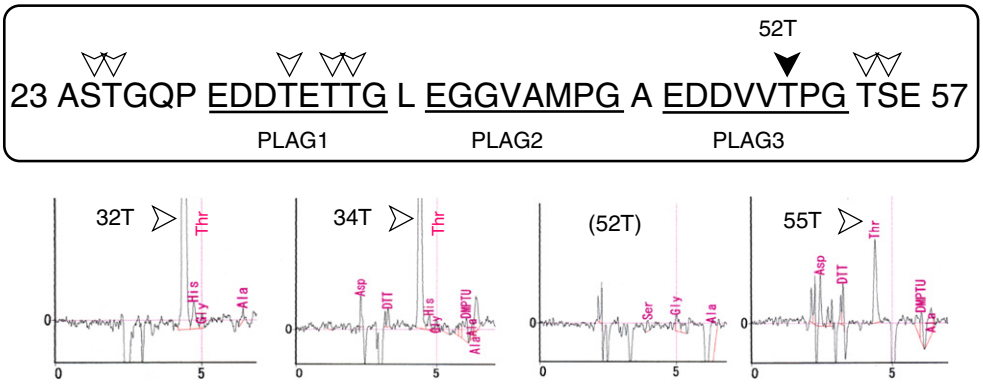

Fig. 4. Determination of glycosylation site of podoplanin. Amino acid sequence of a glycopeptide consisting of Ala23–Glu57 and Edman sequencing chromatographs of Asp-N digested hPod/CHO protein. Glycosylated Thr52 appeared as a gap during peptide sequencing. The same results were obtained using hPod/LN319 protein.

The MS spectrum of the glycopeptide (Ala23–Glu57) showed a signal with *m/z* 4367 that was assigned as [Ala23–GLu57 + $(NeuAc)_2HexHexNAc-H]^-$. The glycan structure of this glycopeptide was characterized as disialyl-corel structure by tandem mass spectrometry, using the same method described above (data not shown). Taken together, these data provided more convincing evidence that the sialyl-corel on the PLAG domain (Thr52) was critical for platelet aggregation-inducing activity.

3.5. Sialylation of functional podoplanin

Human podoplanin-transfected Lec2 cells could not induce platelet aggregation [14]. We purified podoplanin (hPod/Lec2) from podoplanin-transfected Lec2 (Fig. 1A). As shown in Fig. 1B, purified and concentrated hPod/Lec2 induced platelet aggregation, although podoplanin-transfected Lec2 cells did not induce aggregation due to its low concentration on these cell surfaces. Likewise, extremely low concentration of purified hPod/Lec2 did not induce platelet aggregation (Fig. 5). Sialic acid was added to hPod/Lec2 protein using recombinant sialyltransferases. Fig. 5 shows the recovery of platelet aggregation-inducing activity of hPod/Lec2 + ST3GalT (monosialyl-corel), or hPod/Lec2 + ST3GalT + ST6GalNAcT (disialyl-corel) in spite of their extremely low concentration. Western-blot analysis showed that sialylated hPod/Lec2 proteins were not degraded (data not shown). These results confirmed that sialic acid on podoplanin is critical for its platelet-aggregating activity.

In summary, we have clarified the glycan structure of human podoplanin, which is important for its platelet-aggregating activity; the disialyl-corel is linked to a glycosylation site at Thr52 in the PLAG domain of podoplanin. Further investigation is needed to identify the minimum unit of the platelet-activating glycopeptide within podoplanin. We are investigating the target molecule for podoplanin on the platelet membrane. However, the receptor of podoplanin has not been identified yet. The results may be exploited to develop new anti-coagulant therapies.

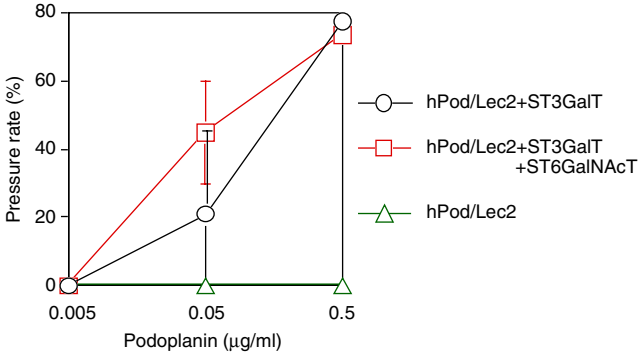

Fig. 5. Recovery of platelet aggregation aggregation-inducing activity. hPod/Lec2 was used as the acceptor substrate for sialylation. hPod/Lec2 + ST3GalT (black circle) means sialyl-corel (NeuAcα2–3Galβ1–3GalNAcα1-) on hPod/Lec2; hPod/Lec2 + ST3GalT + ST6GalNAcT (red square) means disialyl-corel (NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAcα1-) on hPod/Lec2. hPod/Lec2 (green triangle) was treated in the same experimental condition without ST3GalT and ST6GalNAcT. Platelet aggregation was measured using WBA Carna with the screen filtration pressure method. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgements: We thank Ms. Kahori Tachibana, Ms. Sanae Furukawa, Ms. Kozue Hagiwara, Ms. Yuki Tsunoda, and Mr. Noboru Uchiyama for their technical assistance. We are very grateful to Drs. Webster K Cavenee, Naoya Fujita, and Takashi Tsuruo for their generous donation of materials. This work was performed as a part of the Medical Glycomics Project supported by NEDO (New Energy and Industrial Technology Development Organization). This study was supported in part by a grant for Research Fellowships from the Japanese Society for the Promotion of Science (Y. Kato), by Kanae Foundation for Life and Socio-medical Science (Y. Kato), and by the Osaka Cancer Research Foundation (Y. Kato).

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