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Binding of von Willebrand factor cleaving protease ADAMTS13 to Lys-plasmin(ogen)

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The metalloprotease ADAMTS13 affects platelet adhesion and aggregation through depolymerization of von Willebrand factor (VWF) multimers. Identification of ADAMTS13-binding proteins would reveal the hitherto unrecognized mechanisms underlying microvascular thrombus. To identify ADAMTS13-binding proteins, we performed a yeast two-hybrid screen using the Cys-rich and spacer domains of ADAMTS13, the critical regions for the binding and cleavage of VWF, as a bait region. We identified Lys-plasminogen, an amino-terminal truncated form of plasminogen, as the binding protein to ADAMTS13. Intact Glu-plasminogen did not bind to ADAMTS13. Active-site blocked Lys-plasmin bound to ADAMTS13. Domain truncation of ADAMTS13 and elastase digest of plasminogen indicated that the Cys-rich and spacer domains of ADAMTS13 and the kringle 5 and protease domains of plasminogen served as the main binding sites. Biacore measurements revealed that Lys-plasminogen bound to ADAMTS13 with a K_d of $1.9 \pm 0.1 \times 10^{-7}$ M and Glu-plasminogen exhibited a significantly lower affinity to ADAMTS13. Specific activity measurements revealed that ADAMTS13 and Lys-plasmin were still active even after the binary complex was formed. The binding of ADAMTS13 to Lys-plasminogen may play an important role to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

Keywords: ADAMTS13/fibrinolysis/plasminogen/thrombotic thrombocytopenic purpura/von Willebrand factor.

Abbreviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13; APMSF, *p*-amidinophenyl methanesulfonyl fluoride; CUB, complement components C1r and C1s/urinary epidermal growth factor/bone morphogenic protein-1; Glu-Pg, Glu-plasminogen; HRP, horseradish peroxidase; Lys-Pg, Lys-plasminogen; mAb, monoclonal antibody; mini-Pg, mini-plasminogen; VWF, von Willebrand factor.

Platelet thrombus formation is dependent on the multimeric sizes of von Willebrand factor (VWF) under shear stress conditions. VWF multimers are depolymerized by plasma metalloprotease ADAMTS13. Thus, ADAMTS13 regulates the VWF-dependent platelet thrombus formation. Congenital or acquired deficiency of ADAMTS13 can cause thrombotic thrombocytopenic purpura that is characterized with thrombocytopoenia and microangiopathic haemolytic anaemia, sometimes accompanied with transient neurological dysfunction (*1–4*). ADAMTS13 has multiple discrete domains, comprising a metalloprotease domain (M), a disintegrin-like domain (D), a first thrombospondin type-1 repeat (T), a Cys-rich region (C), a spacer domain (S), seven consecutive T repeats and two CUB (Complement components C1r and C1s/urinary epidermal growth factor/bone morphogenic protein-1) domains (*5–7*).

ADAMTS13 cleaves a single specific peptide bond of Tyr^{1605}–Met^{1606} within the A2 domain of VWF under shear stress conditions *in vivo* or under denatured conditions *in vitro*. This restricted substrate specificity can be defined by several structural features in ADAMTS13. The C and S domains in ADAMTS13 play a critical role on the binding and cleavage of VWF, and the S domain seems to be highly important for the recognition of VWF (*8*,*9*). Studies using ADAMTS13 mutants and VWF peptides indicated cooperative and modular interaction of discrete segments of VWF with ADAMTS13 (*10–13*). The crystal structures of the DTCS domains showed three VWF-binding exosites on the linearly aligned discontinuous surfaces of the D, C and S domains (*14*,*15*). Two C-terminal CUB domains are also important for regulation of VWF cleavage *in vitro* as well as *in vivo* (*16–20*). Thus, the interaction between ADAMTS13 and VWF has been intensively investigated; however, the binding proteins for ADAMTS13 are not well known.

Fibrinolytic system in blood is involved in dissolution of blood clots and maintains a patent vascular system. The key component of the fibrinolytic system is plasmin that degrades fibrin clots. Plasmin is

generated from the inactive proenzyme, plasminogen, by cleavage of the Arg561–Val562 peptide bond. Two distinct physiological plasminogen activators, tissue type- or urokinase type-plasminogen activator, convert plasminogen to active plasmin on the fibrin or cell surface. Native plasminogen has N-terminal glutamic acid, designated Glu-plasminogen (Glu-Pg). Lys-plasminogen (Lys-Pg), an amino-terminal truncated form of plasminogen, is formed by the release of a 76-amino acid pre-activation peptide from intact Glu-Pg by the action of plasmin. Because Lys-Pg shows a more open conformation than Glu-Pg, plasminogen activators preferentially cleave Lys-Pg than Glu-Pg. To inhibit the fibrinolytic system, a plasminogen activator inhibitor-1 or α_2-plasmin inhibitor forms an inactive complex with plasminogen activator or plasmin, respectively (*21*).

In the present study, we performed a yeast two-hybrid screen using the critical regions, the C and S domains, for the VWF binding as a bait. The co-immunoprecipitation analysis, the far-western blotting and the Biacore measurement indicated that Lys-Pg is the binding protein to ADAMTS13. ADAMTS13 and Lys-plasmin were active even after the binary complex was formed. The binding of ADAMTS13 to Lys-Pg may play an important role to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

Materials and Methods

Yeast two-hybrid screen

The Matchmaker Two-hybrid System 3 (Clontech, Palo Alto, CA, USA) was used according to the manufacturer's instructions. A fragment encoding the C and S domains of human ADAMTS13 (amino acids 440–685) was used as the bait. cDNA libraries (Clontech) constructed from human liver and brain mRNA (1.3×10^8 and 1.4×10^7 clones, respectively) were screened. Insert DNA of positive clones was sequenced, and the sequence homologies were searched by basic local alignment search tool (BLAST).

Binding of ADAMTS13 to immobilized candidate proteins

The binding of ADAMTS13 (3 μg/ml) to immobilized proteins (9 μg/ml) was examined using microtiter plates. Bound ADAMTS13 to immobilized proteins was detected using anti-ADAMTS13 monoclonal antibody (mAb) WH2-22-1A, which recognizes the disintegrin-like domain (*22*), and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody. Bound HRP activity was detected at 450 nm with a reference wavelength of 650 nm using 3,3′,5,5′-tetramethylbenzidine substrate (KPL, Gaithrburg, MD, USA) and a Multiskan Ascent microplate reader (Thermo, Waltham, MA, USA).

Co-immunoprecipitation analysis of ADAMTS13 with Glu-Pg or Lys-Pg

Human ADAMTS13 with a FLAG tag (ADAMTS13-FLAG) and two mutants, MD-FLAG constituting the M and D domains with the FLAG tag and MDTCS-FLAG constituting the M, D, T, C and S domains with the FLAG tag, were expressed in the culture medium using HeLa cells, as previously described (*8*). Culture medium containing each of those recombinant proteins was incubated with intact Glu-Pg (Calbiochem, Madison, WI, USA) and/or Lys-Pg (Calbiochem) in Tris-buffered saline (TBS: 50 mM Tris, 100 mM NaCl, pH 7.5) and immunoprecipitated with anti-FLAG M2 mAb-immobilized gel (Sigma-Aldrich, St. Louis, MO, USA). After washing with TBS containing 0.5% Tween-20 (TBS-T), proteins were eluted by the FLAG peptide, and subjected to SDS–PAGE for western blotting using anti-FLAG M2 mAb (Sigma) or anti-Pg mAb MAB2596 (R&D Systems, Minneapolis, MN, USA). Alternatively, we used the anti-Pg mAb and protein G-agarose (Sigma) for the co-immunoprecipitation analysis of purified ADAMTS13 (*22*) with Glu-Pg, Lys-Pg or *p*-amidinophenyl methanesulfonyl fluoride (APMSF)-treated Lys-plasmin (Calbiochem). Bound proteins were eluted with 100 mM glycine-HCl, pH 2.5, and then subjected to SDS–PAGE for western blotting using anti-ADAMTS13 mAb WH10, which recognizes the fourth thrombospondin type-1 repeat (*22*) or anti-Pg mAb MAB2596. Immunoblots were probed with HRP-conjugated anti-mouse IgG antibody. Protein bands were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA, USA) on an image analyser LAS3000 (Fujifilm, Tokyo, Japan).

Identification of ADAMTS13 binding region in Lys-Pg

Lys-Pg (0.1 mg) was digested with porcine pancreatic elastase (5 μg; Sigma). The resulting mini-plasminogen (mini-Pg), a functionally active zymogen containing the kringle 5 and protease domains, and fragments containing the kringle 1–4 domains were obtained in the unbound and bound fractions, respectively, using a lysine-Sepharose column (GE Healthcare, Little Chalfont, UK) (*23*). Proteins were subjected to SDS–PAGE for N-terminal sequence analysis and transferred onto polyvinylidene difluoride membranes for far-western blotting. Proteins on the membranes were incubated with ADAMTS13. Bound ADAMTS13 was detected with the HRP-conjugated anti-ADAMTS13 polyclonal antibody (*22*) prepared using Peroxidase Labeling Kit-NH_2 (Dojindo, Kumamoto, Japan) and visualized using Western Lightning Chemiluminescence Reagent Plus on the image analyzer LAS3000.

Lys-Pg binding to ADAMTS13 using Biacore

The binding of Glu-Pg or Lys-Pg to ADAMTS13 was examined using a Biacore 2000 (GE healthcare, Piscataway, NJ, USA). ADAMTS13 was immobilized on a CM5 sensor chip with an amino coupling kit (GE healthcare) according to manufacturer's instructions. Approximately 500–600 resonance units (RU) of ADAMTS13 were covalently attached onto the chip. Lys-Pg (0.05, 0.1, 0.2, 0.4 and 0.8 μM) or Glu-Pg (0.4, 0.8, 1.6 and 3.2 μM) in 50 mM Tris, 100 mM NaCl, pH 7.5, containing 0.005% Tween-20 and 5 mM $CaCl_2$ was injected over the ADAMTS13-immobilized sensor chip at a flow rate of 20 μl/min for 2 min.

The sensor chip was regenerated with 50 mM Tris, 1 M NaCl, pH 7.5, containing 0.005% Tween-20 and 5 mM $CaCl_2$ for 1 min. The dissociation constants (K_d) at the equilibrium were obtained using several ligand concentrations with the BIA evaluation software. Each K_d value was obtained from four or three independent experiments using Lys-Pg or Glu-Pg, respectively.

Activity measurements of ADAMTS13 and plasmin in the complex

ADAMTS13 activity was measured using VWF (*24*) and synthetic fluorogenic substrate FRETS-VWF73 (Peptide Institute, Osaka, Japan) (*25*). For VWF assay, ADAMTS13 (15 ng/ml) was mixed with Glu-Pg (0.1 mg/ml), Lys-Pg (0.1 mg/ml) or bovine serum albumin (0.1 mg/ml) and incubated with guanidine-pretreated VWF multimers (2 mg/ml) for 30, 60 or 120 min at 37°C (*24*). The cleaved fragment with a molecular weight of 200 kDa was assessed by western blotting using HRP-conjugated anti-human VWF polyclonal antibody (DAKO, Carpinteria, CA, USA). For FRETS-VWF73 assay, ADAMTS13 (6.6×10^{-1} nM) was mixed with Glu-Pg (11, 110, 1100 nM) or Lys-Pg (12, 120, 1200 nM). After addition of FRETS-VWF73 (2 μM) to the mixture, increase in fluorescence was measured using Mx3000P System (Stratagene, La Jolla, CA, USA) with 340-nm excitation and 450-nm emission (*25*). The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 min to 10 min using the PRISM software (GraphPad Software, San Diego, CA, USA). The relative activities were estimated from the activity of ADAMTS13 without Glu-Pg or Lys-Pg. To assess the plasmin activity, plasmin (20 nM) was preincubated with ADAMTS13 (40, 80, 200 nM) for 30 min at room temperature followed by the addition of S-2251 (1 mM). Plasmin activity was recorded as a change in absorbance at 405 nm with a reference wavelength of 492 nm during 30 min using the Multiskan Ascent microplate reader.

Results

Yeast two-hybrid screen for ADAMTS13

A yeast two-hybrid screen enabled us to identify more than 500 positive clones. A BLAST search for the insert DNA sequences identified approximately 200 genes, and 36 genes were categorized as membrane or secretory proteins. For further analysis, among these candidate genes, we selected nine secretory proteins that were commercially available or generously donated: Glu-Pg, biglycan (bovine), collagen type I, collagen type III, decorin (bovine), fibrinogen, laminin, histidine-rich glycoprotein and zinc-α2-glycoprotein. We found that ADAMTS13 was bound to immobilized Glu-Pg but not to the others (Fig. 1). The positive clone of human Pg contained a 676-bp cDNA fragment encoding the C-terminal 150 amino acids (amino acid residues 661–810) of the protease domain. ADAMTS13 also bound to Lys-Pg, an amino-terminal truncated form of Glu-Pg (Fig. 1).

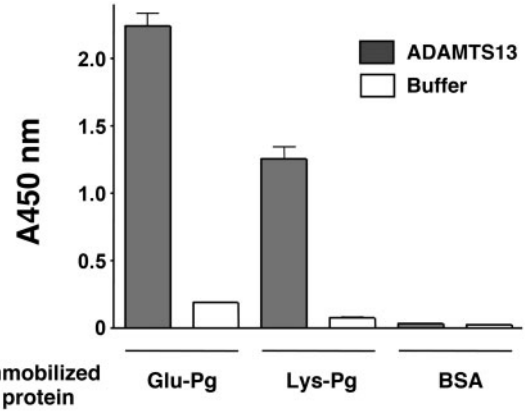

Fig. 1 Binding of ADAMTS13 to immobilized Glu-Pg and Lys-Pg. Microtitre wells were coated with Glu-Pg, Lys-Pg or BSA (each 9 μg/ml) and then incubated with or without ADAMTS13 (3 μg/ml). Bound ADAMTS13 was detected using anti-ADAMTS13 mAb WH2-22-1A (1 μg/ml) and HRP-conjugated anti-mouse IgG (0.25 μg/ml). After incubation with 3,3′,5,5′-tetramethylbenzidine substrate for 20 min, bound HRP activity was detected at 450 nm with a reference wavelength of 650 nm by a Multiskan Ascent microplate reader. The binding was expressed as the mean ± SD ($n = 3$). Grey bar, with ADAMTS13; white bar, without ADAMTS13.

ADAMTS13 binding to Pg

The binding of ADAMTS13 to Pg was examined by co-immunoprecipitation analysis. Anti-FLAG antibody immunoprecipitated ADAMTS13-FLAG with Lys-Pg but not with Glu-Pg (Fig. 2A). Next, anti-Pg antibody was used for the co-immunoprecipitation analysis. Again, ADAMTS13 was co-immunoprecipitated with only Lys-Pg but not with Glu-Pg (Fig. 2B). We found that APMSF-treated Lys-plasmin could be co-immunoprecipitated with ADAMTS13 (Fig. 2C). These results showed that Lys-Pg and Lys-plasmin but not Glu-Pg could bind to ADAMTS13. It is known that Glu-Pg and Lys-Pg have different conformational states in solution (*26*). We assumed that immobilized Glu-Pg had, in part, the conformational change on the plate surface. Microheterogeneity of Pg with or without carbohydrates attached to Asn289 is known (*27*). Doublets of Glu-Pg and Lys-Pg shown in Fig. 2 are likely explained by the carbohydrate difference.

Pg-binding domains in ADAMTS13

Because the C and S domains of ADAMTS13 were used as the bait, the Pg-binding regions would reside in the C and S domains. The co-immunoprecipitation analysis using MD-FLAG and MDTCS-FLAG of ADAMTS13 indicated that both could bind to Lys-Pg but not to Glu-Pg (Fig. 3A). The intensity of bound Lys-Pg was apparently lowest in MD-FLAG and highest in full-length ADAMTS13-FLAG, indicating the gradual loss of affinity in domain truncation. The dose-dependent binding experiments showed that Lys-Pg bound to MDTCS-FLAG at a lower concentration (4 nM) than MD-FLAG (Fig. 3B). Although the Lys-Pg binding to MDTCS-FLAG was saturated at 40 nM, the binding to MD-FLAG was not saturated at the same concentration. The results of the yeast two-hybrid screen and the co-immunoprecipitation

analysis together revealed that the C and S domains in ADAMTS13 are necessary for strong binding to Lys-Pg. Because the M domain of ADMATS13 has two glycosylation sites (*28*), doublets of MD-FLAG might be caused by the difference of carbohydrate. Alternatively, we cannot exclude the possibility of limited proteolysis of MD-FLAG.

ADAMTS13-binding region in Lys-Pg

Elastase has been used for the domain isolation of Pg (*23*), and the resulting fragments containing kringle 1 and 4 domains can bind to lysine-Sepharose. We digested Pg with elastase and unbound and bound fractions for lysine-Sepharose were obtained. Lys-Pg, elastase-digested Lys-Pg and lysine-Sepharose unbound and bound fractions of elastase-digested Lys-Pg were subjected to SDS–PAGE (Fig. 4A) for the far-western blotting using ADAMTS13 as the ligand (Fig. 4B). As a result, ADAMTS13 bound to three bands: Lys-Pg, a 40-kDa fragment and a 32-kDa fragment (Fig. 4B, right). N-terminal sequence analysis revealed that the lysine-Sepharose bound 40-kDa fragment was the kringle 4 and 5 and protease domains (K4-K5-P) and the lysine-Sepharose unbound 32-kDa fragment was mini-Pg, which consists of the kringle 5 and protease domains (K5-P). Thus, we concluded that ADAMTS13 bound to mini-Pg but not to the kringle 1–4 domains. This was compatible with the result of the yeast two-hybrid screen that the positive clone contained C-terminal 150 residues of the protease domain of Pg.

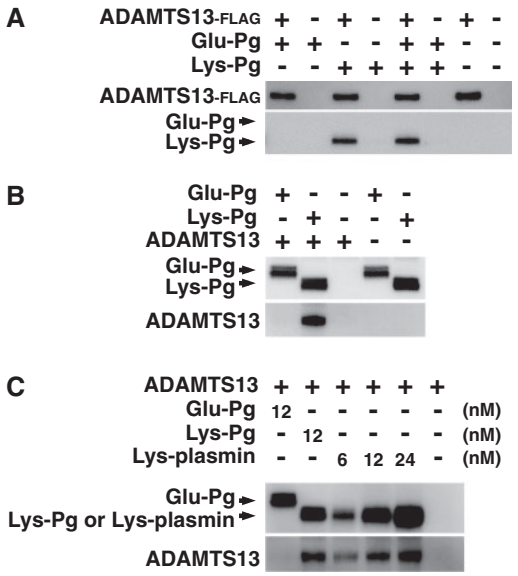

Fig. 2 Binding of ADAMTS13 to Lys-Pg. (A) Co-immunoprecipitation using anti-FLAG mAb. The culture medium containing ADAMTS13-FLAG was incubated with Glu-Pg (100 nM) and/or Lys-Pg (100 nM), and then anti-FLAG mAb-immobilized gel was added to recover bound complexes. Proteins in the complexes were subjected to SDS–PAGE for western blotting using anti-FLAG mAb or anti-Pg mAb. The result is representative of three experiments. (**B** and **C**) Co-immunoprecipitation using anti-Pg mAb. (B) Purified ADAMTS13 (6.7 nM) was incubated with Glu-Pg (12 nM) or Lys-Pg (12 nM), and then anti-Pg mAb was added. Immunocomplexes were subjected to SDS–PAGE for western blotting using anti-Pg mAb or anti-ADAMTS13 mAb WH10. (C) Binding of ADAMTS13 to active-site inhibited plasmin. ADAMTS13 (6.7 nM) was first incubated with Glu-Pg (12 nM), Lys-Pg (12 nM) and APMSF-treated plasmin (6, 12 and 24 nM) and then incubated with anti-Pg mAb. The immunocomplexes were analysed as described in (B). The result is representative of three experiments.

Binding of Pg to immobilized ADAMTS13 using Biacore

ADAMTS13 was immobilized on the sensor chip, and the binding of Glu-Pg or Lys-Pg to ADAMTS13 was measured by recording the changes in surface plasmon resonance upon injection of Pgs using Biacore. We observed that Lys-Pg bound to immobilized ADAMTS13 in the dose-dependent manner (Fig. 5A), whereas Glu-Pg could not significantly bind to ADAMTS13 (Fig. 5B). Lys-Pg exhibited a higher binding affinity to ADAMTS13 with a K_d of $1.9 \pm 0.1 \times 10^{-7}$ M (the mean ± SD) than Glu-Pg with a K_d of $5.5 \pm 2.7 \times 10^{-6}$ M (the mean ± SD).

Plasmin and ADAMTS13 activities in the complex

The C and S domains of ADAMTS13 were necessary for the recognition and cleavage of VWF. Therefore, the Lys-Pg binding to ADAMTS13 may affect the ADAMTS13 activity. We examined the effects of Lys-Pg on the ADAMTS13 activity. We found that Lys-Pg did not affect the ADAMTS13 activity towards the natural substrate VWF (Fig. 6A) and the synthetic

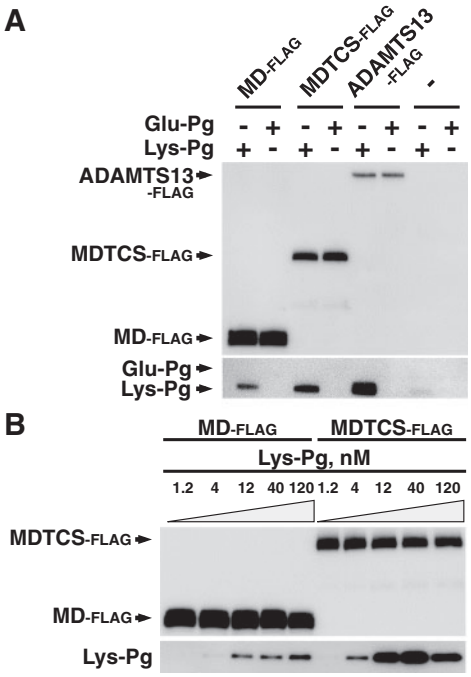

Fig. 3 Binding of ADAMTS13 and its truncated mutants to Glu-Pg or Lys-Pg. (A) Binding of MD-FLAG, MDTCS-FLAG and ADAMTS13-FLAG to Glu-Pg and Lys-Pg. The culture medium containing MD-FLAG, MDTCS-FLAG or ADAMTS13-FLAG was incubated with Glu-Pg (35 nM) or Lys-Pg (35 nM). The complex was immunoprecipitated and then probed with anti-FLAG mAb or anti-Pg mAb. The result is representative of three experiments. (**B**) Dose-dependent binding of Lys-Pg to MD-FLAG and MDTCS-FLAG. Lys-Pg (1.2, 4, 12, 40, 120 nM) was incubated with the culture medium containing MD-FLAG or MDTCS-FLAG. The complex was analysed as described in (A). The result is representative of three experiments.

substrate FRETS-VWF73 (Fig. 6B). The plasmin activity was also not affected by ADAMTS13 even in the 10-fold molar excess of plasmin concentration (Fig. 6C).

Discussion

In this study, we have demonstrated that ADAMTS13 binds to Lys-Pg, the N-terminal truncated form of Pg. This interaction was firstly identified by yeast two-hybrid screen of human liver and brain cDNA libraries using the C and S domains of ADAMTS13 as the bait. This interaction was further demonstrated by the co-immunoprecipitation analysis, the far-western blotting and the Biacore system.

Under physiological conditions, Lys-Pg and Lys-plasmin are not present in circulating blood (*29*). However, in patients undergoing thrombolytic therapy using tissue plasminogen activator, low, but significant, amount of Lys-Pg was detected (*29*). Tissue plasminogen activator can be released from endothelium storage upon venous occlusion, stimulation of epinephrine or desmopressin acetate, and physical exercise. Therefore, under these conditions, Lys-Pg may be locally generated by tissue plasminogen activator and the complex of ADAMTS13 with Lys-Pg might be locally formed, thereby regulating the thrombus formation through VWF cleavage and fibrin degradation.

Physical properties of Glu-Pg and Lys-Pg are quite different. Analysis using small-angle scattering revealed that Glu-Pg has a form with the overall shape of a prolate ellipsoid by interaction between the domains in Pg (*26*). ADAMTS13 can exclusively bind to Lys-Pg but not to Glu-Pg, indicating that ADAMTS13 distinguishes the specific conformation of Lys-Pg. It is known that the conformation of plasmin is resembled to that of Lys-Pg but not Glu-Pg. It is consistent with our result that not only Lys-Pg but also Lys-plasmin can bind to ADAMTS13. Quite recently, the crystal structure of human Glu-Pg has been determined (*30*). The structure clearly showed that seven domains consisting of a Pan-apple domain, five kringle domains and a serine protease domain are loosely clustered in a diamond-shaped zig-zag assembly. Notably, the serine protease domain has a contact with kringle 2 and 4 domains. Although the structure of Lys-Pg remains to be determined, these domain contacts may differ between Glu-Pg and Lys-Pg, resulting in preferable binding of ADAMTS13 to Lys-Pg.

Recently, it was shown that ADAMTS13 is a substrate of plasmin *in vitro* (*31*, *32*). We performed a preliminary experiment as to the ADAMTS13 cleavage with plasmin (data not shown). We found that plasmin cleaved ADAMTS13 into several fragments, and the profile of those fragments was very similar to those previously reported by Crawley *et al.* (*31*) and Hiura *et al.* (*32*). Previous studies showed that the ADAMTS13 activity was progressively decreased by plasmin digestion (*31*, *32*). As for the cleavage sites, plasmin cleaved three peptide bonds, R257-A258 in the metalloprotease domain, R888-T889 in the T4 domain and R1176-R1177 in the T8 domain, but it did not cleave any peptide bonds in the C and S domains

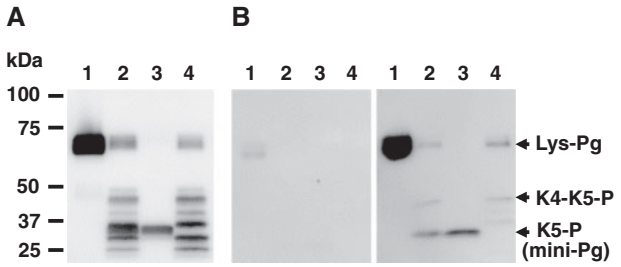

Fig. 4 Binding of ADAMTS13 to mini-Pg. Lys-Pg (lane 1, 1.28 μg protein), elastase-digested Lys-Pg (lane 2, 1.28 μg protein), and lysine-Sepharose unbound (mini-Pg, lane 3, 0.64 μg protein) and bound (several fragments containing kringle 1–4 domains, lane 4, 1.28 μg protein) fractions of elastase-digested Lys-Pg were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. (A) Coomassie Brilliant Blue staining for N-terminal sequence analysis. The N-terminal sequences of 32-kDa (lane 3) and 40-kDa (lane 4) bands were $V^{461}APPP^{465}$ and $V^{374}QDXY^{378}$, respectively, indicating that those were mini-Pg (K5-P) and a fragment consisting of the kringle 4 and 5 and protease domains (K4-K5-P), respectively. (**B**) Far-western blotting. The membrane was incubated without (left) or with (right) ADAMTS13 (4.5 μg/ml). Bound ADAMTS13 was detected by the HRP-conjugated anti-ADAMTS13 polyclonal antibody. The result is representative of three experiments.

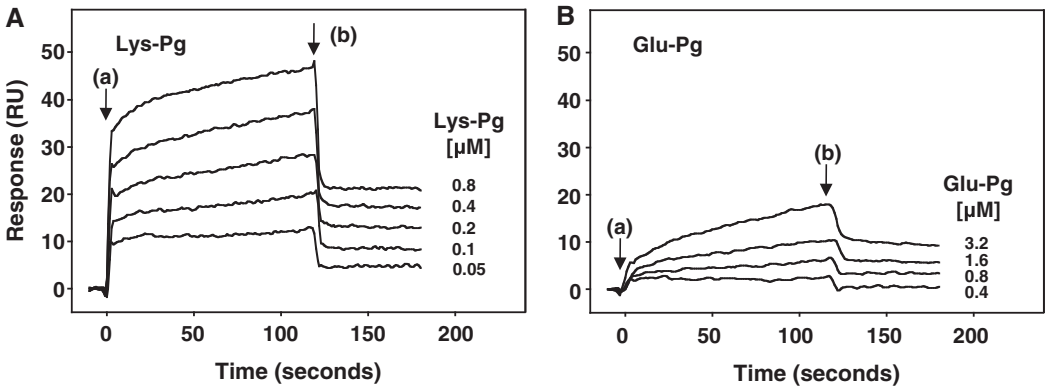

Fig. 5 Binding of Lys-Pg and Glu-Pg to immobilized ADAMTS13 using Biacore. ADAMTS13 was immobilized onto the sensor chip, and Lys-Pg (**A**: 0.05, 0.1, 0.2, 0.4 and 0.8 μM) or Glu-Pg (**B**: 0.4, 0.8, 1.6 and 3.2 μM) was injected over the ADAMTS13-immobilized sensor chip at a flow rate of 20 μl/min for 2 min. The *arrows* indicate the beginning (a) and the end (b) of the application of Pgs. Sensorgrams are shown from a typical experiment, which was repeated at least three times with similar results.

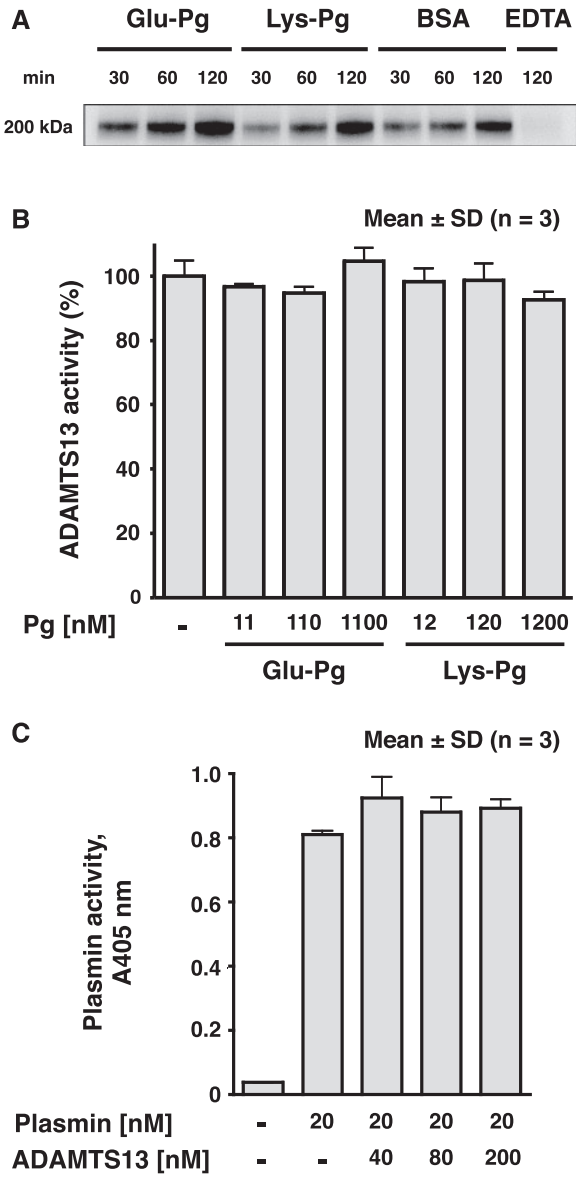

Fig. 6 Plasmin and ADAMTS13 activities in the complex. ADAMTS13 activity was assessed by the appearance of a 200-kDa fragment of VWF using western blotting (**A**) and by FRETS-VWF73 (**B**) as described under the 'Materials and Methods' section. Briefly, for VWF assay, ADAMTS13 was mixed with Glu-Pg, Lys-Pg or BSA and incubated with guanidine-pretreated VWF multimers at 37°C. The cleaved fragment was assessed by western blotting using HRP-conjugated anti-human VWF polyclonal antibody. For FRETS-VWF73 assay, ADAMTS13 was mixed with Glu-Pg or Lys-Pg and was incubated with FRETS-VWF73. Increase in fluorescence was measured with 340-nm excitation and 450-nm emission. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 min to 10 min. The relative activities were estimated from the activity of ADAMTS13 without Glu-Pg or Lys-Pg. The plasmin activity was assessed using S-2251 as substrate (C) as described under the 'Materials and Methods' section. Briefly, plasmin was preincubated with ADAMTS13 followed by the addition of S-2251. Plasmin activity was recorded as a change in absorbance at 405 nm during 30 min.

(*32*). Therefore, the fragments generated from plasmin-digested ADAMTS13 are likely to have intact C and S domains that are necessary for the plasminogen binding.

Fibrin and endothelial proteins, annexin II and α-enolase, bind to Lys-Pg through its lysine-binding site in the kringle domains (*33*, *34*). Since Lys-Pg can bind to cultured endothelial cells in a rapid and reversible fashion via the lysine-binding sites, annexin II and α-enolase are thought to be endothelial receptors for Pg. Interestingly, ADAMTS13 binds to the elastase fragment consisting of the kringle 5 and the serine protease domain of Lys-Pg. Taken together with the result of yeast two-hybrid screen, our observations suggest that the Lys-Pg binding to ADAMTS13 is a novel binding mechanism through the serine protease domain of Lys-Pg. Since the binding site of Lys-Pg to ADAMTS13 is different from that of Lys-Pg to fibrin or endothelial cells, the ADAMTS13–Lys-Pg complex might be anchored to the cells through the kringle domains of the complex. Additionally, we have demonstrated that ADAMTS13 is still active after the complex is formed. Recently, it has been shown that binding of ADAMTS13 to endothelial cells enhances its enzymatic activity (*35*).

In this study, we demonstrated ADAMTS13 binding to Lys-Pg. The physiological role of this binary complex is not clear at present; however, it might contribute to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

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Conflict of interest

K.S. is an employee of Chemo-Sero-Therapeutic Research Institute. The National Cerebral and Cardiovascular Center where T.M. and K.K. (inventors) belong has an awarded patent on the use of reagent, FRETS-VWF73. The other authors state that they have no conflict of interest.

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