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Identification of ADAMTS13 peptide sequences binding to von Willebrand factor

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ABSTRACT

ADAMTS13 cleaves multimeric von Willebrand factor (VWF) to regulate VWF-mediated thrombus formation. To search ADAMTS13 peptide sequences binding to VWF, a λ-phage library expressing various peptides of ADAMTS13 on the surface was screened using VWF either immobilized or in solution under static condition. By the first screening, peptides sharing the C-terminus of spacer domain from Arg^{670} to Gln^{684} (epitope-A) were selected. To explore additional sites, peptide sequences from the first screening were synthesized and added to the second screening. Consequently, Pro^{618} to Glu^{641} (epitope-B) in the middle of spacer domain was obtained from immobilized VWF condition. Synthetic epitope-B peptide inhibited the cleavage of VWF by ADAMTS13, while the synthetic epitope-A peptide did not as efficiently as epitope-B. Elimination of four amino acids from either sides of epitope-B terminus markedly reduced the inhibitory effect. These two sites in the spacer domain may play significant roles in binding to VWF.

Introduction

ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats), a member of the zinc metalloprotease subfamily, cleaves multimeric von Willebrand factor (VWF) to reduce its excessive reactivity to platelet membrane glycoprotein Ib/IX. Under conditions lacking a function of ADAMTS13, VWF excreted from endothelial cells forms ultra-large VWF multimer in blood flow and generates intravascular platelet-rich thrombi, resulting in a predisposed condition of microcirculation disorder, thrombotic thrombocytopenic purpura (TTP). Genetic defects in ADAMTS13 cause Upshaw–Schulman syndrome and inhibitory autoantibodies to ADAMTS13 are commonly seen in acquired idiopathic TTP [1].

ADAMTS13, 1427 amino acid residue long, consists of a signal peptide, a propeptide, a metalloprotease domain, a disintegrin-like domain, a thrombospondin-1 repeat (TSP-1), a cysteine-rich domain, a spacer domain, seven additional TSP-1 repeats, and two CUB domains [2–4]. This protease cleaves VWF between amino acid residues Tyr^{1605} and Met^{1606} in the A2 domain unfolded by shear stress of blood flow *in vivo* [5,6]. Studies on a series of truncated ADAMTS13 proteins at its carboxyl terminus have revealed that a region encompassing from the metalloprotease to the spacer domain is the minimal region required for the proteolysis of VWF under static condition. The disintegrin-like, first TSP-1 repeat, the cysteine-rich and spacer domains interact with VWF and determine the specificity of VWF cleavage under static condition [7–10]. In particular, the spacer domain is considered to play a central role in the specific binding to VWF [9]. Interestingly, most epitopes of autoantibodies detected in acquired idiopathic TTP patients were mapped on the cysteine-rich and spacer domains [8,11,12], suggesting the strong correlation between these domains and VWF.

We tried to search putative peptide sequences of ADAMTS13 binding to VWF using a λ-phage surface display system. This display vector produces a foreign protein or protein fragment as a chimeric fusion to the phage coat proteins [13,14]. A protein fragment library constructed with the vectors can be used for affinity screening of peptides of interest by employing probe molecules immobilized on the surface of solid matrices such as microtiter plates or agarose beads [15–20]. In this study, we made a random peptide fragment (epitope) library of ADAMTS13 by the λ-phage display vector, and subsequently screened the library with VWF either immobilized or in solution under static conditions. From amino acid sequences encoded by the library phage clones collected by the affinity screening, we have successfully explored epitope sites in the spacer domain of ADAMTS13 binding to VWF. The specific binding has also been confirmed by competition assay using synthetic peptides corresponding to the epitope sites.

Materials and methods

Construction of ADAMTS13 epitope library. ADAMTS13 cDNA was kindly provided by Daiich Sankyo Co., Ltd. (Chuo-ku, Tokyo, Japan).

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ADAMTS13 epitope library was constructed as previously described [13–15] with slight modifications. In brief, a plasmid pcDNA3.1 myc-His (Invitrogen, Carlsbad, CA) construct harboring the full length of ADAMTS13 cDNA was treated with DNase I (Takara, Shiga, Japan), and blunted by treatment with T4 DNA polymerase (New England Biolabs, Beverly, MA). After ligating the blunted fragments with Sfi I adaptors, DNA fragments were fractionated by 2.0% agarose (NuSieve; FMC, Rockland, ME) gel electrophoresis. Fragments ranging from 100 to 200 bp were excised and purified by gel extraction kit (Qiagen, Tokyo), then ligated with Sfi I-digested λfooDc vector [19]. The ligation mixture was packaged with MaxPlax (Epicentre Technologies, Madison, WI), and amplified by infection to *Escherichia coli* (*E. coli*) strain Q447 [13]. The resulting ADAMTS13 epitope library consisted of 1.1×10^7 plaque forming units (pfu) of phage clones.

Affinity selection. Library affinity selection was performed as described previously [15–20]. In brief, the library phage was grown with *E. coli* TG1 in CY medium, added with protease inhibitor cocktail (Pierce, Rockford, IL), precipitated with polyethylene glycol (Mw ~8000), then suspended in binding buffer (5% skimmed milk, 0.25% bovine serum albumin (BSA), 0.1% Tween-20, 0.1% sodium azide in PBS). The library expressing ADAMTS13 peptide fragments on the surface was incubated with human VWF (Haematologic Technologies, River Road, VT), 7.5 μg/ml, in phosphate buffered saline (PBS)–0.1% sodium azide either immobilized on microtiter wells or in static solution at 4 °C overnight. For the screening in static solution, bound phage to VWF was collected by protein G beads (Dynabeads, Invitrogen Dynal AS, Oslo, Norway) coated with anti-VWF polyclonal antibody (Dako Japan, Kyoto). After washing three times with binding buffer, twice with washing buffer-1 (5% skimmed milk, 0.5% Tween-20 in PBS) and once with washing buffer-2 (10 mM Tris–HCl; pH 7.4, 5 mM $MgSO_4$, 0.2 M NaCl, 10 mM $CaCl_2$), bound phage was eluted by collagenase (Sigma, Tokyo) and titrated using *E. coli* JM105 with the indicator X-gal. The affinity panning procedure was repeated three times, then the white plaques were picked up randomly and the inserts were analyzed by DNA sequencing. In addition, to evaluate the effect of divalent metal cations on ADAMTS13-VWF binding, 2.5% BSA with or without 5 mM EDTA instead of 5% skimmed milk was also used for the binding. Furthermore, to explore additional sites other than the first screening, peptide sequences obtained from the first screening were synthesized and added to the concentrated phage suspension in binding buffer at final concentrations ranging from 0.2 to 1 mM for the second screening.

Expression of recombinant ADAMTS13 protein. Recombinant ADAMTS13 protein was expressed as previously described [21]. In brief, pcDNA3.1 myc-His harboring ADAMTS13 cDNA was transfected into HEK293 cells using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN). After cultivating for 24 h, the culture medium was replaced with serum-free OPTI-MEM1 (Invitrogen), and further cultivated for 72 h. After the culture medium was collected and condensed, the concentration of recombinant ADMTS13 was measured by enzyme-linked immunosorbent assay (ELISA) (American Diagnostica, Stamford, CT).

Inhibition of ADAMTS13 activity by synthetic epitope peptides. FRETS-VWF73 assay kit was used to measure ADAMTS13 activity according to the manufacture's instruction (Peptide Institute, Osaka, Japan). For the assay, the concentration of recombinant ADAMTS13 protein was adjusted to the level of that in standard plasma (0.7 μg/ml). To measure inhibitory effects of the synthetic peptides on ADAMTS13 activity, the recombinant ADAMTS13 proteins were mixed with the serially diluted synthetic peptides. After the addition of FRETS-VWF73, emitted fluorescence intensity was measured [22].

VWF-cleaving activity of ADAMTS13 in reaction buffer containing urea and $BaCl_2$ was evaluated in the presence of various concentrations of synthetic peptides as described previously [23,24]. In brief, concentrated culture medium containing 0.4 μg/ml of recombinant ADAMTS13 was mixed with 15 μg/ml of purified VWF in reaction buffer (1.5 M urea, 5 mM Tris–HCl; pH 8.0, 10 mM $BaCl_2$, 1.0 mM PMSF), and incubated at 37 °C for 16 h. The reaction was stopped by the addition of EDTA at a final concentration of 10 mM. The samples were subjected to SDS–agarose gel electrophoresis and transferred to PVDF (polyvinyl difluoride) membrane. The patterns of multimeric VWF were immunostained with anti-VWF polyclonal antibody (Dako Japan, Kyoto).

Dissociation constants of epitope peptides for VWF. Dissociation constants of the synthetic epitope peptides for VWF were studied by surface plasmon resonance using a BIAcore 2000 system (BIACORE, Tokyo) at 25 °C according to the manufacture's instruction. In brief, VWF was covalently immobilized on a CM-5 (carboxylated dextran matrix) sensor chip by aldehyde coupling. The synthetic peptide solutions were serially diluted (200–0.39 μM) in HBS-N buffer (10 mM Hepes; pH 7.4, 150 mM NaCl), and injected at a flow rate of 10 μl/min. After each injection, the bound peptide was stripped with 50 mM NaOH for 30 s prior to subsequent injection. For the calculation of kinetic constants, the mode of steady state affinity was used (BIAevaluation software, version 2.1).

Results and discussion

ADAMTS13 peptide sequences binding to immobilized VWF

The ADAMTS13 epitope library was affinity-screened using VWF immobilized on a microtiter plate. Phage clones were randomly picked up and DNA sequences of the phage inserts were analyzed. As a result, almost a half of the picked-up clones exhibited peptide sequences sharing the C-terminus of spacer domain from Arg^{670} to Gln^{684} (termed as epitope-A) (Fig. 1A). Another peptide sequence was frequently seen among the rest clones, however, the peptide was excluded since it did not show ADAMTS13 amino acid sequence because of a frameshift.

To examine the possibility of ADAMTS13 epitope sites for VWF binding other than the epitope-A, two peptide sequences obtained from the first screening, R^{670}PDITFTYFQPKPRQAWV^{687} (PP-a: the shortest peptide from epitope-A sequences) or ETHVPGHWREL HHEAWR (PP-x: irrelevant peptide sequence from the first screening) were synthesized and either of the synthetic peptides were added to the second screening. Surprisingly, all of the picked-up clones from the second screening with PP-a (1 mM) exhibited identical sequence in the middle of spacer domain from Pro^{618} to Glu^{641} (termed as epitope-B) (Fig. 1A). The epitope-B peptide was not obtained from the first screening, and hence we speculate that the addition of PP-a induced the conformational change of immobilized VWF, followed by the exposure of hidden binding site for the epitope-B. No other significant peptide sequences were identified when PP-x (0.2 mM) was added to the second screening. We further repeated affinity selection in the presence of synthetic epitope-B peptide (PP-b: P^{618}SLLEDGRVEYRVALTEDRLPRLE^{641}), or every pairs of synthetic peptides in PP-a, PP-b, PP-x, or all of the three synthetic peptides, however, any significant new epitope sites were not found.

To evaluate the effect of divalent metal cations on the binding of ADAMTS13 epitopes to immobilized VWF, affinity selections were carried out in the presence or absence of 5 mM of EDTA, however, no new epitope site was found (data not shown), suggesting divalent metal cations may not mask other binding sites [9].

ADAMTS13 peptide sequences binding to VWF in static solution

The ADAMTS13 epitope library was next affinity-screened using VWF in static solution. Similar to immobilized VWF, many

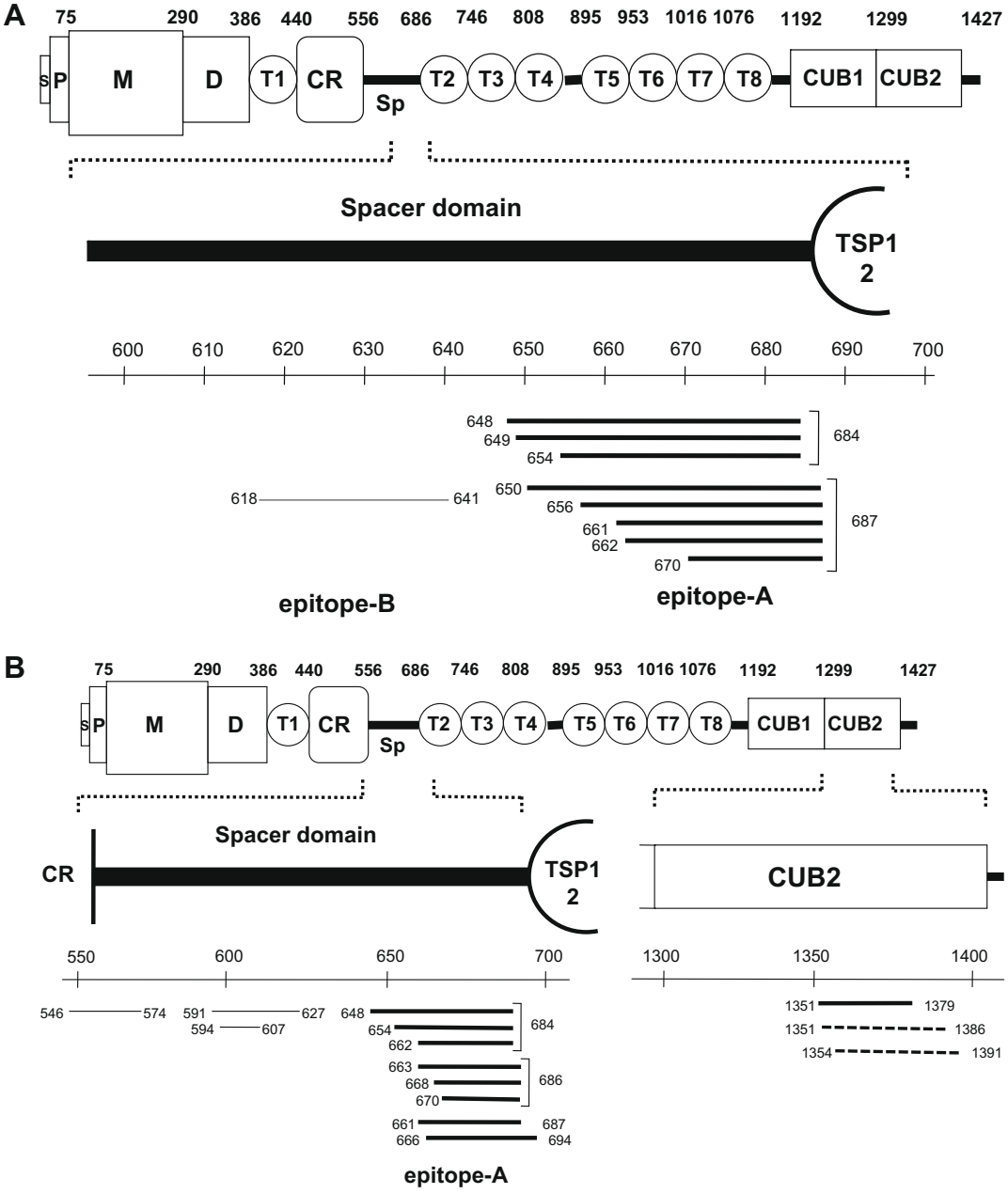

Fig. 1. Structure of ADAMTS13 and epitope sites for VWF binding. Molecular structure of ADAMTS13 is depicted as a chain of domains with the number of the first amino acid residue of each domain on top. Epitope regions encoded by phage clones are shown by horizontal lines with residue numbers of the N- and C-termini. Bald lines show peptide sequences from the first screening. Standard lines and dotted lines show peptide sequences from the second screening. The following abbreviations are used: "S" for signal peptide, "P" propeptide, "M" metalloprotease domain, "D" disintegrin-like domain, "T" thrombospondin-1 repeat, "CR" cysteine-rich domain, "Sp" spacer domain, and "CUB" C1r/C1s, urinary EGF, and bone morphogenetic protein. (A) ADAMTS13 peptide sequences binding to immobilized VWF. (B) ADAMTS13 peptide sequences binding to VWF in static solution.

picked-up clones exhibited epitope-A (Fig. 1B), although less efficiently collected than the immobilized VWF condition. In addition, another same irrelevant peptide sequence was also detected. Previous reports showed that the conformation of VWF is folded and globular in static solution, however, is unfolded under immobilized or shear stress conditions [25,26]. The ADAMTS13 spacer domain is considered to bind to the VWF-A2 domain exposed by the conformational change [27–29]. Our data might suggest that small groups of VWF in static solution are exposing its A2 domain, although the efficiency of binding to epitope-A site is less than immobilized VWF.

The second screening was performed with synthetic peptides PP-a or PP-x. Addition of PP-a revealed only a few clones exhibiting the peptide sequences of N-terminal or middle of spacer domain, although they were not identical to epitope-B (Fig. 1B). This may suggest that VWF in solution could change the conformation less correctly than immobilized VWF upon the epitope-A binding for the following epitope-B binding. When we added PP-x to the second screening in solution, two clones sharing the identical peptide sequence were detected in the latter half of CUB2 domain and the sequence was also detected in one clone from the first screening without the synthetic peptide (Fig. 1B). The overlapping sequence was Asn^{1354} to Trp^{1379} in CUB2 domain, suggesting the possible interaction with VWF in static solution. In previous studies under flow conditions, the CUB1 domain may serve as the docking site for ADAMTS13 to bind VWF [30], and TSP-1 repeats 2–7 and CUB

domains are required for the recognition and cleavage of VWF [31]. Our result may indicate the weak interaction between the CUB2 domain of ADAMTS13 and VWF in static solution, although further investigation should be necessary.

Inhibition of ADAMTS13 activity by epitope peptides

We examined the inhibitory effect of the synthetic epitope peptides (PP-a, PP-b) on ADAMTS13 protease activity using the FRETS-VWF73 assay. As shown in Fig. 2A, the synthetic peptides, at the final concentrations ranging from 25 to 125 μM, inhibited the cleavage of FRETS-VWF73 by ADAMTS13; PP-b had the stronger inhibitory effect than PP-a. To examine the critical peptide length of PP-b required for the inhibition, amino acid residues at either N-terminus or C-terminus of PP-b were removed individually and the synthetic peptides were again assessed for the inhibitory effect on ADAMTS13 activity. The elimination of four amino acid residues from either N-terminus (Fig. 2B) or C-terminus (Fig. 2C) markedly reduced the inhibitory effect of PP-b on ADAMTS13 activity.

The stronger inhibitory effect of PP-b than that of PP-a was also confirmed by SDS–agarose gel electrophoresis analysis of cleavage products of multimeric VWF molecules. As shown in Fig. 3, increased concentrations of PP-b in the reaction buffer inhibited the cleavage of multimeric VWF by ADAMTS13, whereas the inhibitory effect of PP-a was much weaker.

Affinity of epitope peptides for VWF

To compare affinities of the two synthetic epitope peptides, PP-a, PP-b, for VWF, we measured dissociation constants (K_D) using surface plasmon resonance with immobilized VWF on a sensor chip. As a negative control peptide, PP-aR, reverse peptide sequence of PP-a, was synthesized. The synthetic epitope peptides, PP-a (K_D: 4.1×10^{-6}) and PP-b (K_D: 3.1×10^{-7}), had significantly higher affinities for VWF than that of the control peptide PP-aR (K_D: 5.1×10^{-5}); PP-b had the higher affinity for VWF than PP-a. Since epitope-B had an order of magnitude higher affinity for immobilized VWF than that of epitope-A, it is unlikely that the first screening of the library accidentally failed to detect epitope-B.

From these results, we are considering two step interactions between VWF and the spacer domain of ADAMTS13. First the epitope-A sequence binds to and causes VWF conformational change, thereby exposing the buried binding site for the epitope-B. Second the epitope-B sequence bind to the site to stabilize the interactions. Accordingly, we tried to detect the enhancive effects of PP-a on the affinity of PP-b for immobilized VWF or on the inhibition of VWF cleavage by PP-b, using BIAcore system or FRETS-VWF73 assay, respectively. However, we could not obtain any significant effects of PP-a, probably because of the physiologically different conditions such as the concentration of synthetic peptides or the conformation of VWF.

In addition to the spacer domain, furthermore, first TSP-1 repeat and the cysteine-rich domains are also considered as exosites to interact with VWF-A2 domain [32], and the disintegrin-like domain plays a major role in the precise positioning of VWF for cleavage [33], and a monoclonal antibody recognizing the disintegrin-like domain of ADAMTS13 inhibits the protease's binding and catalytic activity to VWF *in vitro* [34]. These indicate that other sites of ADAMTS13 than the spacer domain contribute to the interaction with VWF. During the preparation for this report, crystal structures of noncatalytic domains of ADAMTS13 from the

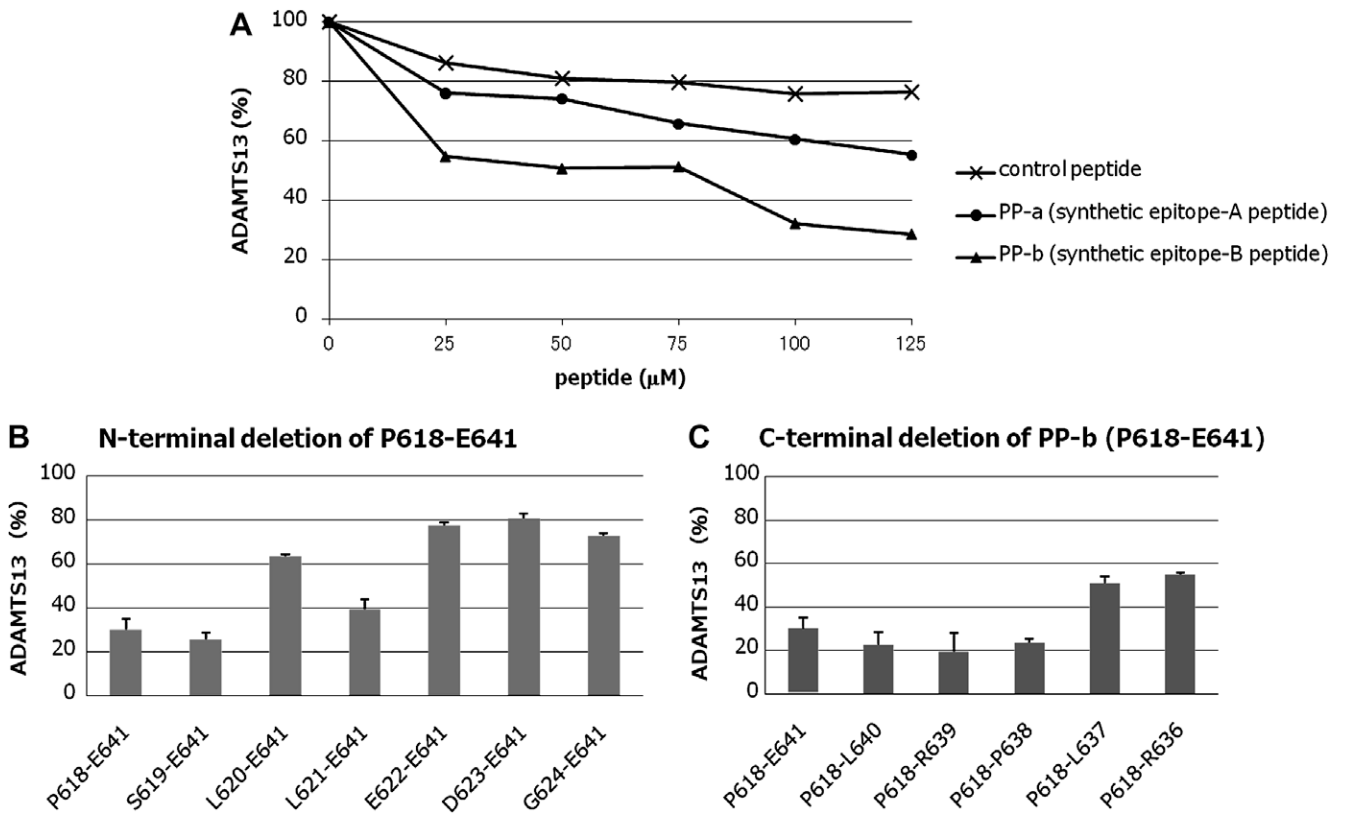

Fig. 2. Inhibition of ADAMTS13 protease activity by synthetic epitope peptides. (A) The recombinant ADAMTS13 (0.7 μg/ml) was mixed with the serially diluted synthetic peptides and FRETS-VWF73 was used as a substrates. Duplicate assays were performed three times, and the data points were the average of the measurements. (B) N-terminal amino acid of PP-b (synthetic epitope-B peptide, 200 μM) was removed individually and the inhibitory effects on ADAMTS13 activity were measured. (C) C-terminal amino acid of PP-b was removed individually and the inhibitory effects on ADAMTS13 activity were measured.

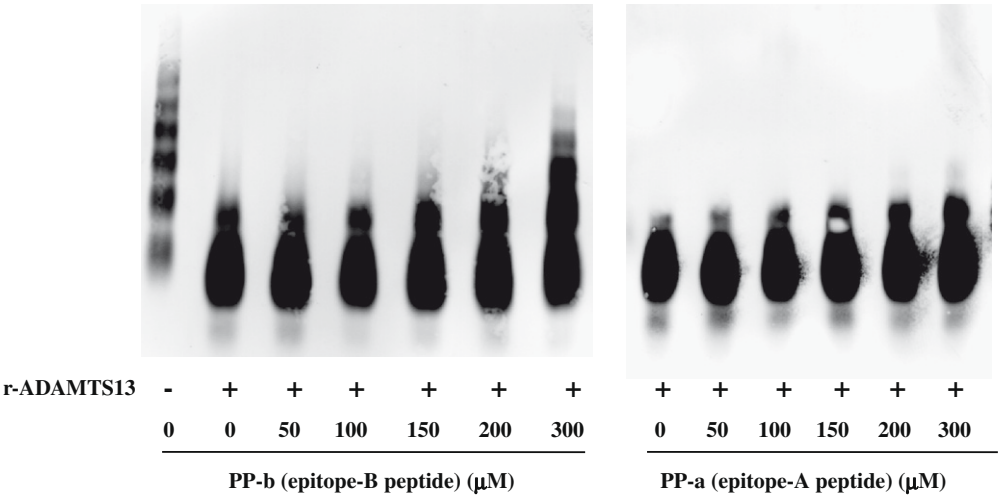

Fig. 3. Inhibition of cleavage of multimeric VWF by synthetic epitope peptides. Recombinant ADAMTS13 and purified VWF with the serially diluted synthetic epitope peptides (PP-a or PP-b) were mixed at indicated concentrations in reaction buffer. After incubation, the samples were subjected to SDS–agarose gel electrophoresis and transferred to PVDF membrane. VWF multimeric pattern was visualized with anti-VWF polyclonal antibody.

disintegrin-like to the spacer were published [35]. They identified three VWF-binding exosites in the disintegrin-like, the cysteine-rich and the spacer domains, respectively. Interestingly, in the spacer domain, epitope-A peptide groups were corresponding to the VWF-binding β9-β10-loop and β10-sheet, and epitope-B peptide sequence included the cysteine-rich – spacer interface, β7-sheet and VWF-binding β7-β8-loop. In our study, it remains to be elucidated why the β-sheet structures were required for the VWF binding in addition to the VWF-binding loops. In particular, we suppose that Arg^{670} to Gln^{684} of epitope-A in β10-sheet plays an important role in the initial interaction and subsequent alteration of VWF conformation in spite of its relatively weak affinity. According to the crystal structure study, residues 1653-1668 of VWF-A2 segment forms amphipathic α-helix and contacts with β9-β10-loop of spacer domain, and hence the subsequent flanking region from Cys^{1669}-Cys^{1670}, which is suggested to play critical role in the unfolding of VWF-A2 [36], or other segments of VWF, may interact with Arg^{670} to Gln^{684} of epitope-A in β10-sheet. Moreover, it should be concerned that all the data in the current study were collected under conditions without shear stress *in vitro*, therefore, additional regions such as TSP-1 repeats or CUB domains may also be involved in the binding *in vivo*. Since the peptide fragments of ADAMTS13 expressed on the phage surface were only about 30–70 amino acids in length, other potential epitopes, particularly conformational ones, may also be missed in the current screening. In addition, ADAMTS13 epitopes containing posttranslational modifications cannot be identified in this screening due to the lack of the modification in *E. coli.*

Conclusions

We explored two epitope sites for VWF binding in the spacer domain of ADAMTS13 using the λ-phage surface display system. Synthetic epitope peptides corresponding to the epitope sites showed both inhibitory effects on the cleavage of VWF by ADAMTS13 and affinity for immobilized VWF, and hence these two peptide sequences may play significant roles in binding to VWF.

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