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A Novel Human Metalloprotease Synthesized in the Liver and Secreted into the Blood: Possibly, the von Willebrand Factor–Cleaving Protease?[1]

Kenji Soejima,*[2] Noriko Mimura,* Masaki Hirashima,* Hiroaki Maeda,* Takayoshi Hamamoto,† Tomohiro Nakagaki,† and Chikateru Nozaki*

**First Research Department and †Blood Products Research Department, The Chemo-Sero-Therapeutic Research Institute, Kumamoto 869-1298*

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We identified a novel metalloprotease, which could be responsible for cleaving the Tyr842-Met843 peptide bond of von Willebrand factor (vWF). This metalloprotease was purified from Cohn Fraction-I precipitate of human pooled plasma by the combination of gel filtration, DEAE chromatography, and preparative polyacrylamide gel electrophoresis in the presence of SDS. The NH_2-terminal amino acid sequence of the isolated protein was: AAGGILHLELLVAVGPDVFQAHQEDTRRY. Based on this sequence, we searched human genomic and EST databases, and identified compatible nucleotide sequences. These results suggested that this protein is a novel metalloprotease, a member of the family of a disintegrin and metalloprotease with thrombospondin type-1 motifs (ADAMTS), and its genomic DNA was mapped to human chromosome 9q34. Multiple human tissue northern blotting analysis indicated that the mRNA encoding this protease spanned approximately 5 kilobases and was uniquely expressed in the liver. Furthermore, we determined the cDNA sequence encoding this protease, and found that this protease was comprised of a signal peptide, a proregion followed by the putative furin cleavage site, a reprolysin-type zinc-metalloprotease domain, a disintegrin-like domain, a thrombospondin type-1 (TSP1) motif, a cysteine-rich region, a spacer domain, and COOH-terminal TSP1 motif repeats.

Key words: ADAMTS, disintegrin-like, metalloprotease, thrombospondin type-1, thrombotic thrombocytopenic purpura, von Willebrand factor, vWF-cleaving protease.

von Willebrand factor (vWF) plays two major roles in hemostasis; *i.e.*, to support platelet adhesion and aggregation to injured blood vessel walls, and to serve as the carrier protein for coagulation factor VIII (*1*). The vWF protein is synthesized in endothelial cells (*2*) and megakaryocytes (*3*) as a pre-pro form with a total of 2,813 amino acid residues including a signal peptide with 22 amino acids. Pro-vWF consists of a 741–amino acid propolypeptide region, and the mature 2,050–amino acid vWF monomer with a molecular mass of 225 kDa. vWF in circulating blood exists as a series of multimers with molecular weights ranging from approximately 500 to 20,000 kDa (*4*). It is known that the highest molecular weight multimeric species have the most potent hemostatic efficacy (*5, 6*). Patients with von Willebrand disease, such as those with the absence of large vWF multimers (possibly due to excess proteolysis), are more susceptible to bleeding events. On the other hand, under shear stress conditions in circulation, even in healthy individuals, vWF becomes more susceptible to proteolysis (*7–10*). It has been reported that a small but consistent proportion of the vWF in normal plasma is composed of 189, 176, and 140 kDa fragments derived from the 225 kDa of vWF monomer *in vivo* (*11*). In 1996, a protease which was responsible for cleaving between amino acid residues Tyr842 and Met843 of vWF was partially purified, resulting in 176 and 140 kDa fragments, and it was suggested that this protease was assumed to be a metalloprotease, since bivalent ions were required to display the protease activity (*12, 13*). Recently, it was also reported that a deficiency of the vWF-cleaving protease is associated with thrombotic thrombocytopenic purpura (TTP), of which the characteristic pathological feature was the hyaline thrombi that occlude capillaries and precapillary arterioles (*14*). Therefore, the vWF-cleaving protease was thought to play an important role in the regulation of the functions of vWF. However, the biochemical properties of the vWF-cleaving protease have not yet been characterized. Here we report the identification of the vWF-cleaving protease and its gene cloning.

MATERIALS AND METHODS

Materials—Sephacryl S-500 HR, S-300 HR, HiTrap

[1] The nucleotide sequence reported in this paper has been submitted to the DDBJ with the accession number AB069698.

[2] To whom correspondence should be addressed.

Tel: +81-968-37-3100, Fax: +81-968-37-3616, E-mail: soejima@kaketsuken.or.jp

Abbreviations: ADAMTS, a disintegrin and metalloprotease with thrombospondin type-1 motif; TSP1, thrombospondin type-1; vWF, von Willebrand factor; RACE, rapid amplification of cDNA ends; TTP, thrombotic thrombocytopenic purpura; EST, expressed sequence tag.

DEAE Sepharose Fast Flow, and molecular weight markers for SDS-PAGE were purchased from Amersham Pharmacia Biotech (Little Chalfont, England). A preparative SDS-PAGE system (Biophoresis™ III) was purchased from the ATTO Corporation (Tokyo). Restriction enzymes and LA Taq™ polymerase with GC Buffer were obtained from Takara Shuzo (Shiga). All other chemicals were of analytical grade or the highest quality commercially available, and were obtained from Wako Pure Chemical Industries (Osaka). The cryoprecipitate and Cohn Fraction-I precipitate were generously provided from the blood production department of the Chemo-Sero-Therapeutic Research Institute (Kumamoto).

Preparation of vWF—vWF was purified by gel filtration of cryoprecipitate derived from human pooled plasma according to the method of Furlan *et al.* (*12*) with some modifications. Briefly, a sample of 2 g of cryoprecipitate was dissolved in 20 ml of 50 mM Tris-HCl buffer containing 0.01% Tween-80 and 100 mM NaCl, pH 7.4. The sample was filtrated with a 1.2 μm membrane filter and subjected to Sephacryl S-500 HR gel filtration column (2.6 × 90 cm) equilibrated with 50 mM Tris-HCl buffer containing 0.01% Tween-80 and 100 mM NaCl, pH 7.4. The high molecular weight fractions estimated by SDS-PAGE were pooled. The protein concentration was determined using bovine serum albumin as a standard with protein assay reagent (Bio-Rad, California, USA), based on Bradford's method (*15*).

Assay of vWF-Cleaving Activity—The assay was performed using the method reported by Furlan *et al.* (*12*) with some modifications. Briefly, 50 μl of test sample was preincubated with 10 mM $BaCl_2$ for 5 min, and then 50 μl of purified vWF (approximately 200 μg/ml) was mixed and dialyzed overnight using a circular dialysis membrane (Millipore, Massachusetts, USA) against 5 mM Tris-HCl, pH 8.0, containing 1.5 M urea at 37°C. Subsequently, the reaction mixtures were subjected to SDS-PAGE according to Laemmli's method (*16*) using a 5% gel under reduced or non-reduced conditions. The gel was stained with Coomassie Brilliant Blue R-250.

Purification of vWF-Cleaving Protease—A sample of 12 g of the frozen Cohn Fraction-I precipitate derived from human pooled plasma was thawed overnight at 4°C, and dissolved in 120 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and 0.05% sodium azide (buffer A), followed by stirring at 37°C for 2 h. The sample was filtrated through a 0.8 μm membrane filter and subjected to Sephacryl S-300 HR column (5 × 90 cm) equilibrated with buffer A. After discarding 600 ml of the eluate, fractions (10 ml each) were collected, and active fractions were then pooled. Saturated ammonium sulfate solution was added to the pooled fraction at up to 33% saturation and incubated overnight at 4°C. The precipitate was obtained by centrifugation and dissolved in buffer A. The above gel filtration and precipitation by ammonium sulfate procedures were repeated twice more. Then, the precipitated active fraction was dissolved in 50 mM Tris-HCl, pH 7.1, containing 50 mM NaCl (buffer B), and dialyzed overnight against buffer B at 4°C. After dialysis, the sample was applied to a DEAE-Sepharose column (1 ml bed volume) and eluted with 5 ml of 0.25 M NaCl. A sample of 5 ml of the eluted fraction was concentrated to 0.5 ml by centrifugation using Centricon™ YM-30 (Millipore). The concentrate was subjected to non-reduced preparative SDS-PAGE in an 8% gel (16 × 25 mm). Approximately 0.5 ml each of fractions was collected. The active fractions were pooled and concentrated again using Centricon™ YM-30. Preparative SDS-PAGE was performed again on the pooled active fractions.

NH_2-Terminal Amino Acid Sequencing—Sequence analysis was performed with an Applied Biosystems 492 protein sequencer (Applied Biosystems, Tokyo), or was commissioned to APRO Science (Tokushima).

Computer Analysis Based on NH_2-Terminal Amino Acid Sequence—Using the SWsrch Search Service (tswn program, http://www.dna.affrc.go.jp/htdocs/SWsrch/index.html), we scanned the DNA database on the experimentally determined NH_2-terminal amino acid sequence.

DNA Sequencing—DNA sequencing was performed using a PCR procedure employing the fluorescent dideoxynucleotide dye terminator method and a 373A automated sequencer (Applied Biosystems).

Northern Blotting Analysis—Gene specific primers for PCR were designed, based on the sequence obtained from the computer search (Fig. 2). The sequences were as follows: 5′-GCTGCAGGCGGCATCCTACACCTGGAGCTG-3′ (forward primer of primer 1), and 5′-CCCAATCTCATG-GGCAATGGT-3′ (reverse primer of primer 2). PCR was performed using Human Universal QUICK-Clone™ cDNA (Clontech, California) as a template with the above primers. The cycling parameters were 35 cycles of 96°C for 20 s and 65°C for 4 min. The amplified DNA fragment was cloned into pCR2.1 using a TA cloning™ kit (Invitrogen, California), and the sequence was confirmed. Finally, *Eco*RI digested fragment was used to make a probe for northern blotting analysis. Subsequently, northern blot immobilized poly(A)$^+$ RNA from various human tissues (Clontech) was hybridized with the [α-^{32}P]dCTP-labeled probe, which was generated by a random priming kit (BcaBEST™, Takara) using the DNA fragment described above as a template, in the presence of 50% formamide at 42°C, overnight, and washed in the absence of formamide at 65°C, followed by autoradiographic exposure at –80°C for 2 weeks, or in the case of β-actin probe, exposed at –80°C for 4 h.

Determination of the cDNA Sequence Encoding vWF-Cleaving Protease—5′-Rapid amplification of cDNA ends (5′-RACE) reaction was performed using human liver Marathon-Ready™ cDNA (Clontech) according to the manufacturer's instructions. Briefly, the first PCR amplification was performed using the AP1 primer provided with the kit and the gene specific reverse primer (primer 3 or 4, Fig. 2). The second PCR was performed using the first PCR products as a template with the internal primers, AP2 primer provided with the kit and the gene specific reverse primer (primer 1 or 3, Fig. 2). PCR products were cloned into pCR2.1 using a TA cloning™ kit (Invitrogen) and sequenced. 3′-RACE was performed using 3′-Full RACE Core Set (Takara) and human liver poly(A)$^+$ RNA (Clontech) as a template, according to the manufacturer's instructions. After reverse transcription, PCR was performed using the Adaptor Primer provided with the kit and the gene specific forward primer (primer 2, Fig. 2). The cycling parameters were as follows: 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min, followed by 72°C for 7 min.

RESULTS AND DISCUSSION

Purification and Determination of NH_2-Terminal Amino

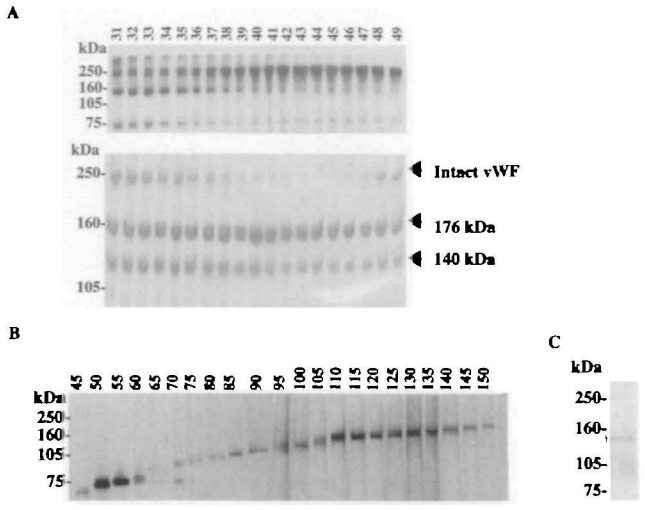

Fig. 1. **Purification of the vWF-cleaving protease.** In panel A, the upper gel shows SDS-PAGE of the fractions obtained from the 3rd gel filtration in an 8% gel under non-reduced conditions. The lower gel shows reduced SDS-PAGE analysis of vWF-cleaving activity (see "MATERIALS AND METHODS"). When each of the eluted fractions (No. 31 to 49) were incubated with intact vWF, two bands resulting from cleavage of intact vWF polypeptide appeared in the active fractions, which corresponded to the 176 and 140 kDa fragments (COOH-terminal and NH_2-terminal regions of vWF), and the band of intact vWF disappeared. The vWF-cleaving activity was detected in fractions No. 37–47. In panel B, the DEAE-eluted sample was subjected to preparative electrophoresis in an 8% polyacrylamide gel in the presence of SDS. Fractions were analyzed by analytical SDS-PAGE in an 8% gel under non-reduced conditions, and the gel was stained with silver. The numbers of the fractions were noted above each lane. The vWF-cleaving protease activity was detected in fractions No. 80-90. In panel C, the purified vWF-cleaving protease was run in a 7.5% SDS-PAGE under reduced conditions, and the gel was stained with silver.

Acid Sequence of vWF-Cleaving Protease—A sample of 12 g of Cohn Fraction-I precipitate dissolved in buffer A (containing approximately 2.4 g of protein) was first subjected to Sephacryl S-300 HR gel filtration. The fractions showing vWF-cleaving protease activity were pooled and precipitated with 33% saturated ammonium sulfate. The precipitate was dissolved in buffer A and subjected to the same column again. These procedures were repeated twice more. Figure 1A shows the results of non-reduced SDS-PAGE of

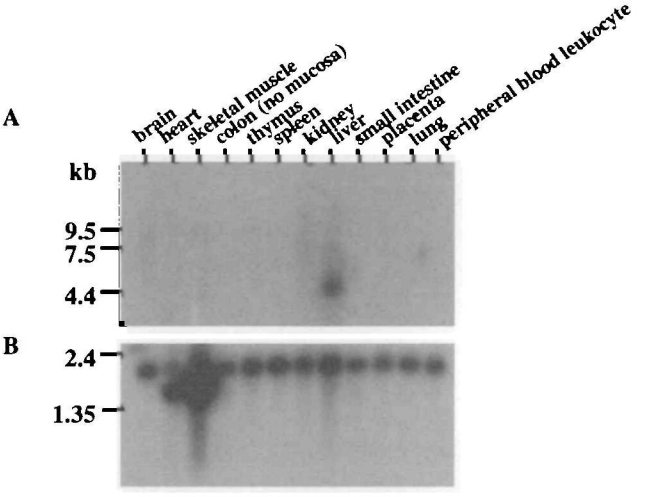

Fig. 3. **Northern blotting analysis of the vWF-cleaving protease expression in various human tissues.** RNA markers are shown at the left, and tissue origins are indicated above each lane. First, hybridization with the cDNA encoding the metalloprotease domain of the vWF-cleaving protease was performed (A), and the filter was subsequently hybridized with a human β-actin probe to control for the loading of RNA (B).

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GCT GCA GGC GGC ATC CTA CAC CTG GAG CTG CTG GTG GCC GTG GGC CCC GAT GTC TTC CAG
A   A   G   G   I   L   H   L   E   L   L   V   A   V   G   P   D   V   F   Q
Primer 1
GCT CAC CAG GAG GAC ACA GAG CGC TAT GTG CTC ACC AAC CTC AAC ATC GGG GCA GAA CTG
A   H   Q   E   D   T   E   R   Y   V   L   T   N   L   N   I   G   A   E   L
                        (R) Primer 3
CTT CGG GAC CCG TCC CTG GGG GCT CAG TTT CGG GTG CAC CTG GTG AAG ATG GTC ATT CTG
L   R   D   P   S   L   G   A   Q   F   R   V   H   L   V   K   M   V   I   L

ACA GAG CCT GAG GGT GCT CCA AAT ATC ACA GCC AAC CTC ACC TCG TCC CTG CTG AGC GTC
T   E   P   E   G   A   P   N   I   T   A   N   L   T   S   S   L   L   S   V

TGT GGG TGG AGC CAG ACC ATC AAC CCT GAG GAC GAC ACG GAT CCT GGC CAT GCT GAC CTG
C   G   W   S   Q   T   I   N   P   E   D   D   T   D   P   G   H   A   D   L
                        Primer 4
GTC CTC TAT ATC ACT AGG TTT GAC CTG GAG TTG CCT GAT GGT AAC CGG CAG GTG CGG GGC
V   L   Y   I   T   R   F   D   L   E   L   P   D   G   N   R   Q   V   R   G

GTC ACC CAG CTG GGC GGT GCC TGC TCC CCA ACC TGG AGC TGC CTC ATT ACC GAG GAC ACT
V   T   Q   L   G   G   A   C   S   P   T   W   S   C   L   I   T   E   D   T

GGC TTC GAC CTG GGA GTC ACC ATT GCC CAT GAG ATT GGG CAC AGC TTC GGC CTG GAG CAC
G   F   D   L   G   V   T   I   A   H   E   I   G   H   S   F   G   L   E   H
                        Primer 2
GAC
D
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Fig. 2. **Amino acid and nucleotide sequences of the NH_2-terminal metalloprotease region of the vWF-cleaving protease.** The identified nucleotide sequence and the deduced amino acid sequence are shown. The experimentally determined NH_2-terminal amino acid sequence from residue 1 to 29 is shown in boldface. There was a difference in the amino acid sequence at position 27. The experimentally determined amino acid at position 27 (arginine) is shown in parentheses. The primer sites used for preparation of the probes for northern blotting and RACE PCR are underlined.

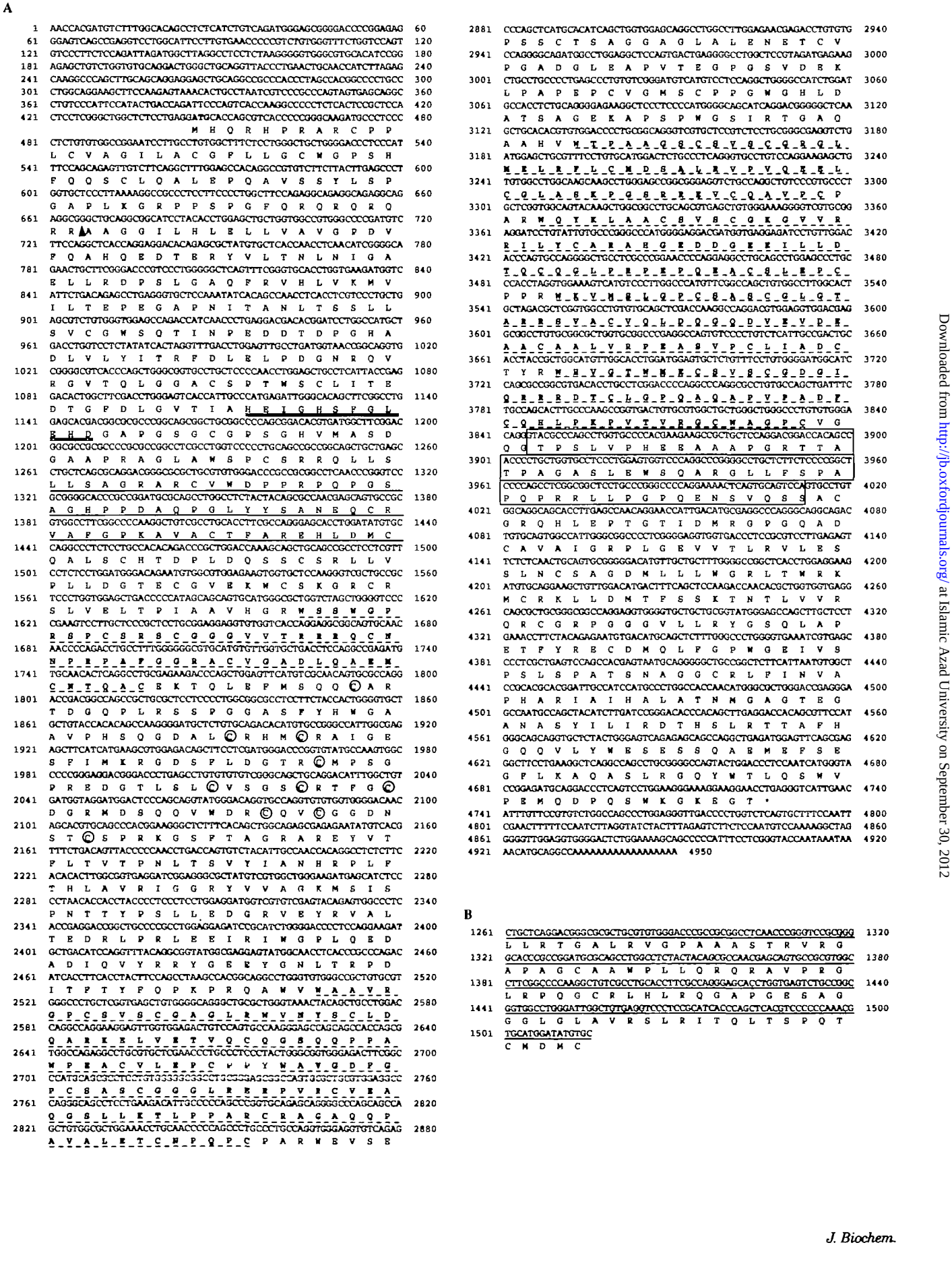

Fig. 4. **Nucleotide sequence of the human vWF-cleaving protease cDNA and its deduced amino acid sequence.** In panel A, the deduced entire amino acid sequence is shown below the DNA sequence. The numbers refer to the positions of nucleotides. The first ATG sequence (Met) is shown in boldface. The predicted furin cleavage site is marked with an arrowhead. The putative zinc-binding motif is double underlined and the disintegrin-like region is underlined. Each thrombospondin type-1 motif is underlined with a broken line and shown in boldface. Cysteine residues in the cysteine-rich region are circled. The region deleted by an alternative splicing at a COOH-terminal region is boxed. The termination codon is indicated by an asterisk. In panel B, nucleotide and amino acid sequences are shown, resulting from alternative splicing at the site of a disintegrin-like domain (underlined).

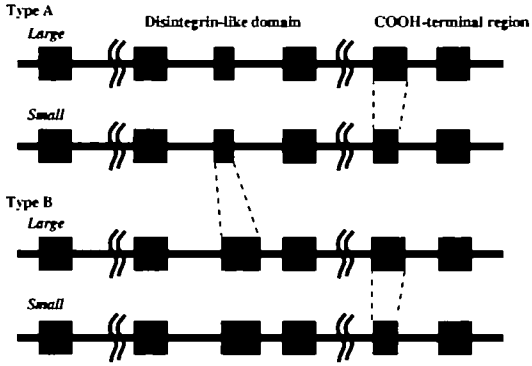

Fig. 5. **Schematic representation of the four isoforms genetically isolated.** We genetically isolated four isoforms resulting from alternative splicings at the sites of a disintegrin-like domain and a COOH-terminal region. Coding regions of the exons are expressed by the closed boxes. The variation between Type A and B originates from a difference of splicing patterns at the site of a disintegrin-like domain. Furthermore, designation as either a large or small molecule depended on the deletion of a COOH-terminal alternative spliced region. Putative amino acid compositions of the four isoforms after cleaving by furin are as follows: Type A large molecule (the major isoform genetically isolated) is composed of 1,353 residues, type A small molecule (the 2nd major isoform genetically isolated) is composed of 1,297 residues, type B large molecule (rare isoform genetically isolated) is composed of 1,378 residues, and type B small molecule (rare isoform genetically isolated) is composed of 1,322 residues.

the eluted fractions obtained from the 3rd cycle (Fig. 1A, upper), and their vWF-cleaving activity (Fig. 1A, lower). As shown in the lower section of Fig. 1A, when each of the eluted fractions (No. 31 to 49) was incubated with intact vWF, two bands appeared at the active fractions and its activity was inhibited in the presence of 10 mM EDTA (data not shown). The result of NH_2-terminal amino acid sequence analysis of the 176 kDa band showed the sequence of Met-Val-Thr-Gly-Asn, corresponding to the 843rd–847th residues of human vWF, and thus suggested that the two bands were generated by cleaving the peptide bond between the residues Tyr842 and Met843 along the polypeptide chain of intact vWF. In Sephacryl S-300 HR gel filtration, as shown in Fig. 1A, the peak of the protease activity (fraction No. 37 to 47) appeared with a major protein band, which showed a molecular weight of approximately 250 kDa, estimated under non-reduced SDS-PAGE. The active fractions were pooled (containing approximately 20 mg of protein). Furthermore, the sample (containing approximately 10 mg of protein), following HiTrap DEAE chromatography, was subjected to non-reduced preparative SDS-PAGE in an 8% gel, since the cleaving activity remained even after treatment with fractionation by PAGE in the presence of SDS. As shown in Fig. 1B, in non-reduced preparative SDS-PAGE, the peak of activity was detected in fraction No. 80 to 90. Furthermore, these active fractions were pooled, concentrated, and subjected to 2nd cycle preparative SDS-PAGE, and the active fractions were pooled. Finally, the purified vWF-cleaving protease showed a faint single band with a molecular weight with a molecular weight of approximately 140 kDa, estimated under reduced analytical SDS-PAGE (Fig. 1C). Then, after concentration of the purified protease by centrifugation using Centricon™ YM-30, the NH_2-terminal amino acid sequence was determined to be as follows: AAGGILHLELLVAVGPDVFQA-HQEDTRRY.

Computer Analysis Based on NH_2-Terminal Amino Acid Sequence—We performed computational analysis, based on the partial NH_2-terminal amino acid sequence of the purified vWF-cleaving protease (AAGGILHLELLVAV), and identified compatible nucleotide sequences from a human genomic database [three clones from chromosome 9q34 (clone 213M24, clone 107G20, and clone RP11-244N20) were identified with accession numbers AC002099, AC002355, AL158826]. Furthermore, after a search of the EST database, using the sequence of genomic clones, several EST clones were identified (accession numbers AI346761, AJ011374, *etc.*). These results suggest that this protein contains a metalloprotease domain [containing a zinc-binding motif: HEIGHSFGLEHD (Fig. 2)], a disintegrin-like domain and TSP1 domains, and that this protein belongs to the ADAMTS family (*17, 18*). We also found that the genomic DNA encoding this protein is located in human chromosome 9q34.

Northern Blotting Analysis—We cloned the DNA fragment containing the sequence shown in Fig. 2 using a PCR method. Subsequently, we performed northern blotting analysis using the cloned gene as a probe. The results indicated that the mRNA encoding this protease spanned approximately 5 kilobases and was uniquely expressed in the liver (Fig. 3).

Sequencing of cDNA Encoding Human vWF-Cleaving Protease—Finally, we determined the complete cDNA sequence by the rapid amplification of cDNA ends (RACE) method, using human liver cDNA as a template. As predicted, this protein was a new member of the ADAMTS family. The deduced entire amino acid sequence of the protease obtained from the cDNA sequence included a signal peptide, a proregion (unusually shorter than the other members of the ADAMTS family), followed by the putative furin cleavage RQRR site, a reprolysin-type zinc-metalloprotease domain, a disintegrin-like domain, TSP1 motif, a cysteine-rich region, a spacer domain, and six COOH-terminal TSP1 motif repeats (Fig. 4). Furthermore, as shown in Fig. 4, A and B, two alternative splicing sites were detected (at the site of a disintegrin-like region and a COOH-terminal region, respectively), and thus, four alternative splicing forms were genetically isolated (Fig. 5). In addition, when the cDNA sequence was compared with that of the genomic clone 107G20 with accession number AC002355, it was found that the entire open reading frame was comprised in 29 exons and spanned approximately 40

kilobases on human chromosome 9q34.

In conclusion, we have determined the cDNA sequence of a novel metalloprotease as a new member of the ADAMTS family, which has a high likelihood of being responsible for cleaving at the site of Tyr842-Met843 of the vWF molecule.

Note Added in Proof: Recently, two independent groups simultaneously reported the partial NH_2-terminal amino acid sequence of purified vWF-cleaving protease in BLOOD by The American Society of Hematology (*19, 20*). These sequences were in good agreement with that reported here.

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