ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 478–488

BBRC

www.academicpress.com

Protein engineering and properties of human metalloproteinase and thrombospondin 1☆

Ping Wei,[a] Yun-Ge Zhao,[b] Li Zhuang,[a] Douglas R. Hurst,[b] Steve Ruben,[a] and Qing-Xiang Amy Sang[b,*]

[a] *Human Genome Sciences Inc., Rockville, MD 20850, USA*

[b] *Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, Chemistry Research Building DLC, Room 203, Tallahassee, FL 32306-4390, USA*

Received 19 March 2002

Abstract

This work generated many truncated proteins and Glu^{385} to Ala (E^{385}/A) mutants of the human metalloproteinase and thrombospondin 1 (METH-1 or ADAMTS1) and specific antibodies. METH-1 was an active endopeptidase and both the metalloproteinase and the disintegrin/cysteine-rich domains were required for the proteinase activity. A point mutation at the zinc-binding site (E^{385}/A) abolished the catalytic activity. METH-1 protein function may be modulated through proteolytic cleavage at multiple sites. One 135 kDa species had an NH_2-terminal sequence of L^{33}GRPSEEDEE. A species at 115 kDa and some other protein bands began with F^{236}VSSHRYV^{243}, indicating that METH-1 proenzyme might be activated by a proprotein convertase such as furin by cleaving the R^{235}–F^{236} peptide bond. This cleavage was not an autocatalytic process since the E^{385}/A mutants were also processed. Furthermore, a 52 kDa band with an NH_2-terminal sequence of L^{800}KEPLTIQV resulted from the digestion between the first and the second thrombospondin 1-like motifs in the spacer region of the extracellular matrix-binding domains.

Keywords: ADAMTS1; METH-1; Disintegrin and metalloproteinase; Zymogen activation; Mutagenesis; Mammalian expression system; Catalytic residue; Antibody; Structure–function relationship; α2-Macroglobulin

Disintegrin and metalloproteinases (ADAMs, metallospondin 1) represent a new family of gene products that show a significant sequence similarity to snake venom metalloproteinases and disintegrins [1,2]. Most of the ADAMs are comprised of the following domain structures: a signal peptide, a propeptide domain, a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an epidermal growth factor (EGF)-like domain, a transmembrane region, and a cytoplasmic tail [3–5]. More than 30 genes encoding the proteins of the ADAM family have been identified in different species, but the biological functions of most members are still unclear. Their domain structures suggest that they have many potential functions during physiological and pathological processes, including proteolysis, adhesion, fusion, and intracellular signaling. The proteins have been implicated in a variety of important processes, such as degradation of type IV collagen, sperm-egg and muscle cell binding and fusion, myogenesis, osteogenesis, spermatogenesis, tumor cell adhesion, signal transduction, the shedding of tumor

☆ *Abbreviations:* Ab, antibody; ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-like motifs; α2-M, α2-macroglobulin; Brij-35, polyoxyethylene lauryl ether; CM, conditioned media; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; EST, expressed sequence tag; Fc, C-terminal fragment (constant region) of the immunoglobulin G; METH-1, protein-1 with metalloprotease and thrombospondin domains; MMPs, matrix metalloproteinases; MS, mass spectrometry; RP-HPLC, reverse-phase high performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TACE, tumor necrosis factor-α converting enzyme; TBS, tris-buffered saline; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor-α; TSP1, thrombospondin-1.

* Corresponding author. Fax: +1-850-644-8281.
E-mail address: sang@chem.fsu.edu;
http://www.chem.fsu.edu/editors/sang/sang.htm (Q.-X. Amy Sang).

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00255-3

necrosis factor α (TNF-α), and angiogenesis inhibition [5–10].

Disintegrin and metalloproteinase with thrombospondin (TSP)-like motifs-1 (ADAMTS1) are new members of the ADAM family, which was originally identified by differential display analysis as a gene highly expressed in the murine colon 26 cachexigenic tumor [11]. The human ortholog of ADAMTS1 was also named METH-1 due to the novel combination of metalloprotease and thrombospondin domains [10]. Like other typical ADAMs, the amino-terminal half-region of ADAMTS1 consists of propeptide, metalloproteinase, and disintegrin/cysteine-rich domains that share sequence similarities to snake venom metalloproteinases.

The domain organization of the carboxyl-terminal half of ADAMTS1 is completely different from other ADAMs. Instead of the EGF-like domain, transmembrane region, and cytoplasmic tail, ADAMTS1 has three TSP type 1 motifs that are found in thrombospondins 1 and 2 [10–12]. These TSP type 1 motifs of METH-1/ADAMTS1 are functional in binding to heparin and have angio-inhibitory activity [10,13–15]. Analyses of deletion mutants have revealed that the carboxyl-terminal spacing region and the three TSP type 1 motifs are responsible for anchoring to the extracellular matrix (ECM) [13]. Recently, Kuno et al. [16] reported that mouse ADAMTS1 protein is proteolytically active based on its capacity to form a covalent complex with α2-macroglobulin (α2-M). More recent results showed that ADAMTS1 is essential for normal growth, fertility, and organ morphogenesis and function [14] and is able to cleave the Glu^{1871}–Leu^{1872} bond within the chondroitin sulfate attachment domain of aggrecan [17]. In addition, ADAMTS1 has a significant role in the proteolytic events of the ovulation process [18,19]. Furthermore, GON-1, a metalloproteinase that controls gonadogenesis by remodeling the basement membrane in *Caenorhabditis elegans*, also demonstrates a structural similarity to ADAMTS1 [20]. All of these findings suggest that ADAMTS1 may be an active enzyme involved in angiogenesis, inflammation, fertility, and organ morphogenesis.

In this study, we created different ADAMTS1/METH-1 domain construct expression vectors (Fig. 1), expressed the constructs in human kidney 293T cells, and purified the expressed METH-1 proteins. We also designed and synthesized peptides according to protein sequences in different domains of METH-1 and used these peptides to immunize rabbits and obtain specific polyclonal IgG. By performing an α2-M binding assay, we found that the disintegrin/cysteine-rich domain of ADAMTS1/METH-1 is required for its enzymatic activity. The mutant proteins (E385/A) did not show any activities in the same assay suggesting that Glu^{385} may be a catalytic residue of the enzyme.

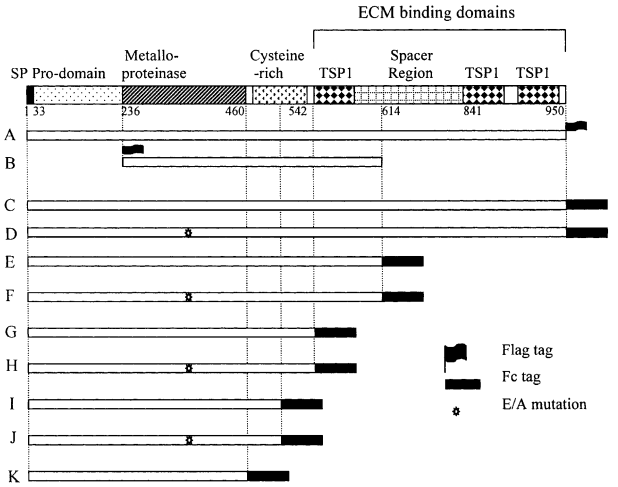

Fig. 1. Schematic diagram of the domain organization and the different constructs of human METH-1. The domain labels are: SP, signal peptide; TSP1, thrombospondin type 1 motif; ▬, pFLAG tag; ▬, pC4Fc tag; ✲, constructs with E385/A mutation in the metalloproteinase domain. A–K: the different constructs of METH-1. A: The pFLAG-residues 1–950 with FLAG tag at its C-terminal; B: The pFLAG-residues 236–614 with FLAG tag at its N-terminal. C–K are pC4Fc:residues 1–950, pC4Fc:residues 1–950E/A, pC4Fc:residues 1–614, pC4Fc:residues 1–614E/A, pC4Fc:residues 1–544, pC4Fc:residues 1–544E/A, pC4Fc:residues 1–510, pC4Fc:residues 1–510E/A, and pC4Fc:residues 1–460, respectively. All the pC4Fc constructs have the Fc tags at their COOH-terminals.

Materials and methods

Construction of expression vectors. Many chemicals and reagents used in the experiments were the same as described [21]. Human METH-1/ADAMTS1 cDNA clone was identified from a human heart library by screening the human cDNA database of expressed sequence tags (ESTs) as described [10]. The full-length cDNA of METH-1 was amplified by polymerase chain reaction (PCR) and cloned into the mammalian expression vector pFLAG-CMV-5a at *Not*I and *Asp*718 sites to create pFLAG-CMV-5a-METH-1 with the FLAG tag at its C-terminal. The pFLAG-METH-1 (residues 236–614) has the FLAG tag at its N-terminal (Fig. 1). Constructs pC4-METH-1-Fc contain the full-length METH-1 (residues 1–950), residues 1–614, 1–544, 1–510, and 1–460, respectively (Fig. 1), fused to human IgG1 Fc domain at its C-terminus. The E^{385} to A mutants were also constructed with the Fc tag at their C-terminal.

Cell culture and transfection. The 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, LTI, Rockville, Maryland) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. The 293T cells were transfected with expression vectors containing different METH-1 constructs (Fig. 1) using Lipofectamine Plus 2000 reagent according to manufacturer's instruction (LTI). Briefly, 5×10^6 cells (50–80% confluent) in a 15-cm dish were incubated with 90 μl Lipofectamine, 60 μl Plus, and 12 μg DNA in DMEM without serum and antibiotics. After incubation at 37 °C, 5% CO_2, and 95% air for 3–5 h, the cell media were replaced with 25 ml DMEM with or without serum and the conditional media were collected after 3–5 days.

METH-1 protein purification. FLAG-tagged METH-1 proteins were purified from the transient transfection supernatant of 293T cells. Briefly, 12 μg mammalian expression construct pFLAG-METH-1 DNA (residues 1–950, 236–614) was incubated with 90 μl Lipofect-

amine and 60 μl Plus reagent (LTI) for 15–30 min. Then the mixture was added to 5 million 293T cells in 50–80% confluence and incubated at 37 °C in 5% CO_2 for 5 h. The transfection reagent was removed and replaced with DMEM. Conditioned media were collected 48–60 h later, spun and filtered to remove any debris, and loaded on an anti-FLAG M2 affinity gel column. The FLAG-tagged protein was purified according to manufacturer's instructions (Sigma). The purified protein was in 1× TBS (100 mM Tris–HCl, pH 7.5, 150 mM NaCl), 100 μg/ml FLAG peptide, and 10% glycerol. The METH-1-Fc fusion proteins were affinity purified on a Protein A–agarose affinity column according to manufacturer's instructions (Sigma Chemical, St. Louis, MO).

Protein N-terminal sequencing. The purified METH-1 proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to ProBlott polyvinylidene difluoride membranes using CAPS buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid, pH 11, 0.005% SDS) [22]. Proteins were visualized by staining with Coomassie brilliant blue R-250 solution and excised fragments were sequenced. The N-terminal sequencing was performed by Dr. Umesh Goli at the Biochemical Analysis, Synthesis, and Sequencing Service Laboratory in the Department of Chemistry at Florida State University (Tallahassee, FL).

Antigen peptide syntheses. The specific peptides corresponding to the unique sequences in the different METH-1 domains were synthesized and purified according to our previous report [23]. The sequence selected from the metalloproteinase domain is Cys^{316}–Asn–Trp–Gln–Lys–Gln–His–Asn–Pro–Pro–Ser–Asp–Arg–Asp–Ala–Glu–His–Tyr–Asp^{334}–NH_2 (peptide no. 332) and from the disintegrin/cysteine-rich domain is Gly^{462}–Thr–Ser–Tyr–Asp–Ala–Asn–Arg–Gln–Phe–Gly–Glu– Asp–Ser–Lys–His^{481}–NH_2 (peptide no. 333) (Fig. 9). Thus, the antibodies (Abs) against peptide nos. 332 and 333 are called pAb332 and pAb333, respectively. The molecular masses and molar extinction coefficients at 280 nm of these two peptides were calculated using genetics Computer Group (GCG) programs [5,21,23]. Their molecular masses are 2340.4 and 2291.4 Da, respectively, and their molar extinction coefficients at 280 nm are 7030 and 1340 $M^{-1} cm^{-1}$, respectively. The sequence homology comparisons of each peptide were also performed against the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm at the National Center for Biotechnology Information web site. Peptides with greater than a 45% level of identity were found to be those of human and mouse METH-1/ADAMTS1 and no other peptides were found. Thus, it is unlikely that the Abs against these peptides would cross-react with other proteins. These peptides were synthesized by Dr. Umesh Goli at the Biochemical Analysis, Synthesis, and Sequencing Service Laboratory in the Department of Chemistry at Florida State University (Tallahassee, FL). The purity of these peptides was verified by reverse-phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) (data not shown).

Antibody production and purification. According to our protocol previously reported for producing and characterizing anti-tissue inhibitor of metalloproteinase-4 Ab [24], the pre-immunization sera were collected from the New Zealand white rabbits 4 weeks before the initial injection. The peptide antigens (one milligram of each) were coupled to Keyhole Limpets Hemocyanin (Sigma Chemical) with 2% glutaraldehyde in 0.2 M phosphate buffer at a pH of 7.3. The conjugated peptide was emulsified in the complete Freund's adjuvant (Sigma Chemical, St. Louis, MO) for initial injection. The rabbits were boosted with the same peptide emulsified in the incomplete Freund's adjuvant every 4–5 weeks. The blood was collected 9 days after each boost. The sera were purified following the instructions of ImmunoPure IgG (Protein A) Purification Kit (Pierce Chem.). Briefly, the serum was diluted with binding buffer and added to the equilibrated Protein A column. The column was washed with 15 ml ImmunoPure IgG binding buffer, followed by elution with 5 ml ImmunoPure IgG Elution Buffer. The concentration of IgG in the elution fractions was monitored by UV spectroscopy at 280 nm. Protein A elution fractions were desalted by flowing through an Excellulose column, followed by washing with TBS buffer. The final purified IgG concentration was calculated by multiplying OD_{280} and 0.8 mg/ml. The pre-immune IgG for each rabbit was also purified for a negative control.

Enzyme-linked immunosorbent assay (ELISA). An enzyme-linked immunosorbent assay (ELISA) was used to assess the potency and specificity of the rabbit polyclonal Abs according to our methods [24]. Polyvinyl micro-ELISA plates were coated with either 50 μl/well of purified METH-1 protein (residues 1–950) or 50 μl/well of different concentrations of the synthetic peptide antigen in borate-buffered saline. Alkaline phosphatase-conjugated goat-anti-rabbit IgG (Sigma Immuno Chemicals, St. Louis, MO) was used as the secondary Ab. *p*-Nitrophenyl phosphate (1 mg/ml) in 0.5 M $MgCl_2$, buffered by 1.0 M diethanolamine buffer at a pH of 9.8 was used as the substrate. After incubation, the plates were read at 405 nm with a Titertek Multiscan MC-340 automatic microplate reader. The different peptide antigens and anti-METH-1 Abs were used to test the specificities and cross-reactivities of these Abs. Pre-immune-IgGs of different Abs were tested as controls.

Western blotting. Western blotting was performed according to standard procedure [24]. Briefly, samples were mixed with an equal volume of 2× Laemmli's sample buffer with 2-mercaptoethanol, heated at 100 °C for 5 min, and subjected to 10% SDS–PAGE. Proteins were transferred onto nitrocellulose membranes. The membranes were blocked overnight in 5% bovine serum albumin–phosphate-buffered saline (BSA–PBS) and then incubated with an anti-FLAG M2 monoclonal Ab (Eastman Kodak), anti-human IgG1 Ab, or our polyclonal Abs, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG polyclonal Abs (Amersham Pharmacia Biotech). After washing with TBS buffer, the bound horseradish peroxidase-conjugated Abs were detected by immunoblotting using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

Binding assay of α2-macroglobulin (*α2-M*). Binding assays of α2-M with different constructs of METH-1 proteins were performed according to the methods described [16,21]. Briefly, recombinant FLAG-tagged METH-1 and METH-1-Fc proteins were prepared by using a 293T cell transient expression system as described above. The cell culture supernatant containing ADAMTS1/METH-1 proteins or the purified METH-1 proteins was mixed with an equal volume of α2-M solution (final concentration of 0.25 U/ml) (Boehringer–Mannheim, Germany) and incubated at 37 °C for 15 h. The reactions were terminated by the addition of an equal volume of 2× Laemmli's sample buffer (60 mM Tris–HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol). The α2-M: METH-1 complexes were analyzed by Western blotting using anti-FLAG M2 monoclonal Ab, anti-human IgG1 Ab, or our polyclonal Abs.

Results

Specificity and potency of the METH-1 polyclonal Abs

The Abs against the METH-1 metalloproteinase domain peptide (pAb332) and disintegrin domain peptide (pAb333) were characterized by ELISA (Fig. 2; data for anti-disintegrin domain Ab not shown). The optimal dilution range for anti-metalloproteinase domain Ab was from 1:100 to 1:800. The specificity of the Abs was determined by testing their cross-reactivity with human ADAM19, matrilysin (matrix metalloproteinase-7, MMP-7), endometase (matrilysin-2, MMP-26), and some other metalloproteinases. At protein concentra-

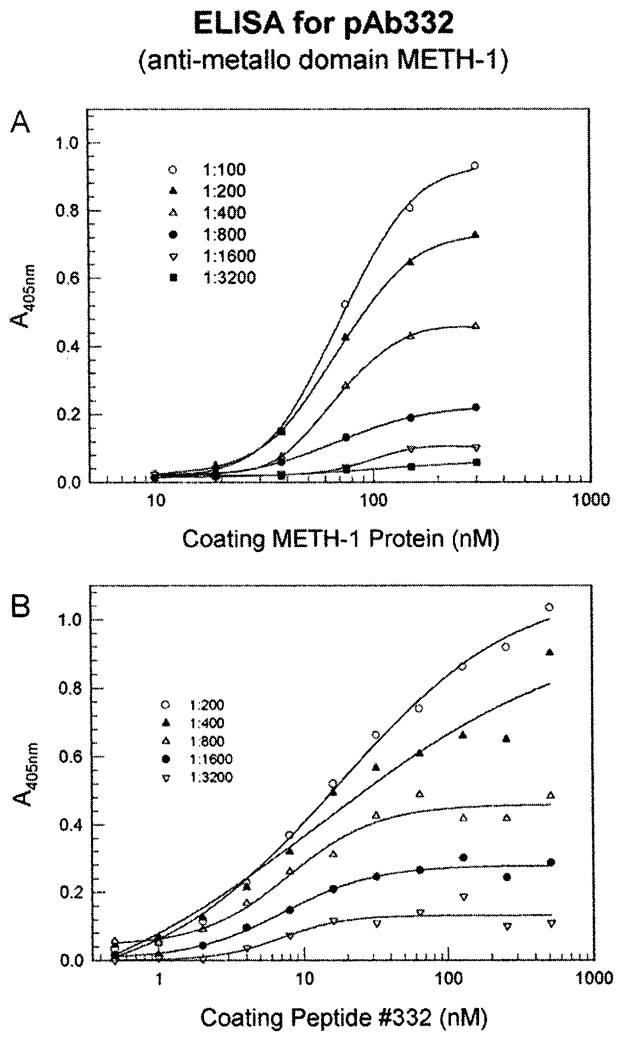

Fig. 2. Characterization of the anti-METH-1 metalloproteinase domain peptide Ab (pAb332) against METH-1 by ELISA. ELISA plates were coated with various concentrations of purified full-length METH-1 (residues 1–950) (A) and METH-1 metalloproteinase domain peptide (residues 316–334) (B). The primary Ab was the rabbit IgG against human METH-1 metalloproteinase domain peptide (residues 316–334). It was purified using the ImmunoPure IgG (Protein A) Purification Kit. The Ab dilutions are indicated in the diagram. The secondary Ab was goat anti-rabbit IgG conjugated with alkaline phosphatase.

tions up to 500 nM and primary Abs dilutions of 1:200, no cross-reactivity was detected (data not shown). The preimmune IgGs of the same rabbits did not react with the peptide antigens (data not shown). The synthesized peptide sequences were utilized in a search and sequence alignment analysis performed against all the current protein data banks by using basic local alignment search tool (BLAST) programs (Bethesda, MD, http://www.ncbi.nlm.nih.gov). The sequences identified were those of human and mouse ADAMTS1/METH-1. This indicated that our polyclonal Abs may only cross-react with mouse METH-1, however, not with other proteins.

Purification of the METH-1 proteins and determining the protein N-terminal sequences

All of the purified METH-1 proteins were revealed by SDS–PAGE, followed by Coomassie blue R-250 staining (Figs. 3A and B). The purified METH-1 (residues 1–614)-Fc had two major bands, one at 78 kDa and another at 32 kDa (Fig. 3A, lane 4). The protein NH_2-terminal sequencing results showed that the 78 kDa had the sequence FVSSHRYV, which is immediately downstream of the proprotein convertase cleavage site of $RKKR^{235}$–$F^{236}VSSHRYV^{243}$ located at the beginning of the METH-1 metalloproteinase domain. The 32 kDa had a sequence of KSSDKTHT, the human IgG1 Fc fragment. The METH-1 (residues 1–544)-Fc showed two bands at 70 and 32 kDa (Fig. 3A, lane 6). The N-terminus of the 70 kDa band was FVSSHRYV and that of the 32 kDa band was KSSDKTHT from the Fc. The E^{385} to A mutant METH-1 (residues 1–614)-Fc, E^{385} to A mutant METH-1 (residues 1–544)-Fc, E^{385} to A mutant METH-1 (residues 1–510)-Fc, METH-1 (residues 1–510)-Fc, and METH-1 (residues 1–460)-Fc were shown only as one major band for each construct at 78, 70, 68, 68, and 60 kDa, respectively (Fig. 3A, lanes 5, 7, 8, and 9; 3B, lanes 4 and 5). The N-terminal sequence results showed that all of the major bands had the sequence FVSSHRYV (Figs. 1 and 9). These results indicated that the prodomains of these METH-1 proteins might be processed by a proprotein convertase such as furin and they were not autocleaved by the METH-1 protein itself because the catalytically inactive mutants were also processed.

Coomassie blue staining of the purified pC4-METH-1-Fc protein revealed bands at 135, 115, 52, 47, and 32 kDa (Fig. 3B, lane 2). The NH_2-terminal sequences of those major protein bands were determined by microsequencing (Fig. 9). The NH_2-terminal sequence of the 135 kDa band was LGRPSEEDEE corresponding to L^{33} of the beginning of the propeptide domain. The 115 kDa band was FVSSHRYV, which is generated by cleavage between R^{235} and F^{236}. The N-terminal sequence of the 52 kDa polypeptide was L^{800} KEPLTIQV. The 32 kDa band has an N-terminal sequence of KSSDKTHT and is a human IgG1 Fc fragment. The N-terminus was blocked for the 47 kDa band and no sequence was obtained. The full-length E to A mutant METH-1-Fc had major bands at 135, 115, and 32 kDa (Fig. 3B, lane 3). The N-terminal sequences of the three bands were identical to those of the wild type full-length METH-1-Fc, respectively. The pFLAG-CMV5a-METH-1 (residues 1–950) protein revealed major bands at 115, 86, and 21 kDa. The 21 kDa band had an N-terminal sequence that starts at L^{800}, L^{800}KEPLTIQV (data not shown). Those data demonstrate that most of the METH-1 proteins were processed by a proprotein convertase to the mature/activated enzymes; however,

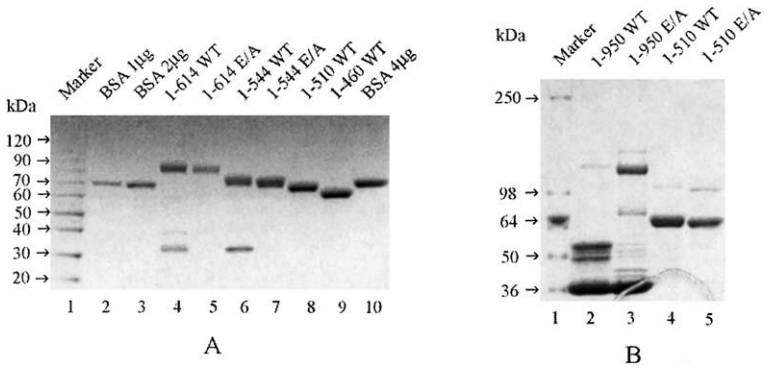

Fig. 3. The purified METH-1 proteins of the different pC4Fc constructs. All of the recombinant METH-1 proteins were separated on 10% SDS–PAGE and stained using Coomassie blue. (A) The proteins are METH-1:residues 1–614 (lane 4), 1–544 (lane 6), 1–510 (lane 8), 1–460 (lane 9), and the METH-1 mutants: residues 1–614E/A (lane 5), 1–544E/A (lane 7). 1, 2, and 4 μg BSA were added in lanes 2, 3, and 10, respectively. (B) Coomassie blue staining results of pC4Fc: METH-1 (residues 1–950) and (residues 1–510) proteins. The wild type and mutant full-length METH-1 (residues 1–950) are shown in lanes 2 and 3, respectively. METH-1 (residues 1–510) and residues 1–510E/A are shown in lanes 4 and 5, respectively.

small amounts of unprocessed proenzymes were also detected in the cell culture media (CM).

Two forms of METH-1 proteins

The 293T cells were transfected with different METH-1-Fc expression vectors and cultured in DMEM (Life Technologies, LTI, Rockville, Maryland) as described in Materials and methods. All of the METH-1 (residues 1–614, 1–544, 1–510, and 1–460) proteins had two forms: a relatively high band (cellular form) and a low band (secreted form). The major bands in the CM are of the secreted form (Fig. 4, lanes 1, 3, 5, and 7), while those in the cell lysates are of the cellular form (Fig. 4, lanes 2, 4, 6, and 8). The 32 kDa bands in the CM phases (Fig. 4, lanes 1, 3, 5, 6, and 7) were Fc fragments according to the N-terminal sequence. The higher molecular mass form found in cell lysate may be the proenzyme and the lower molecular mass forms detected mostly in the cell CM may be the activated and/or C-terminal truncated forms as demonstrated by the above N-terminal sequencing results.

Comparison of the polyclonal antibody and anti-human IgG1 antibody

The METH-1 (residues 1–510)-Fc protein was prepared by a 293T cell transient expression system and purified as described in Materials and methods. The purified METH-1 (residues 1–510)-Fc was detected as a single band (68 kDa) under reducing conditions and a 105 kDa band under non-reducing conditions by Coomassie blue staining (Fig. 5A, lanes 2 and 3). The METH-1 protein was also detected by immunoblotting using an ECL system. The purified pre-immune IgG did not cross-react with any band (Fig. 5B, lanes 1 and 2). The anti-METH-1 metalloproteinase domain peptide

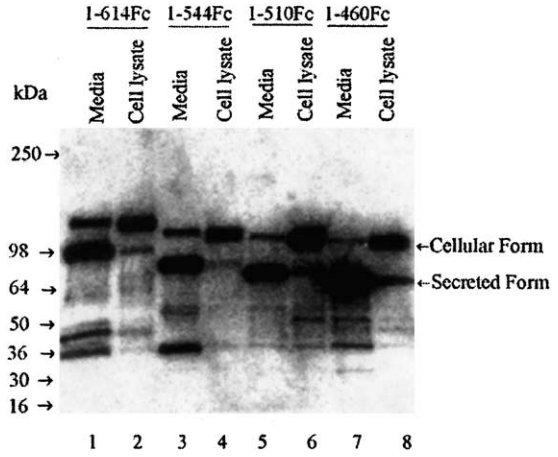

Fig. 4. The distribution of METH-1 proteins in the 293T cell media and cell lysate by immunoblot using anti-IgG1 Ab. The 293T cells were transfected with different METH-1-Fc expression vectors and cultured in DMEM (LTI) as described in Materials and methods. The CMs were collected after 3–5 days. The cells were scraped and lysed. The samples were separated by 10% SDS–PAGE and transferred onto a nitrocellulose membrane. The blot was probed with the anti-human IgG1 Ab and the target proteins were detected by immunoblotting using an ECL system. Lanes 1, 3, 5, and 7 are conditioned cell media and lanes 2, 4, 6, and 8 are cell lysates for METH-1 (residues 1–614), (residues 1–544), (residues 1–510), and (residues 1–460), respectively.

Ab (pAb332) (Fig. 5B, lanes 3 and 4), anti-METH-1 disintegrin domain peptide Ab (pAb333) (Fig. 5B, lanes 5 and 6), and the anti-human IgG1 Ab (hIgG) (Fig. 5B, lanes 7 and 8) recognized the METH-1 (residues 1–510), while only the anti-human IgG1 Ab recognized the control TR7-Fc (Fig. 5B, lane 8).

Formation of METH-1:α2-M complexes in the cell media

The complex formation of proteinases with α2-M is dependent on their endopeptidase activities. The cell

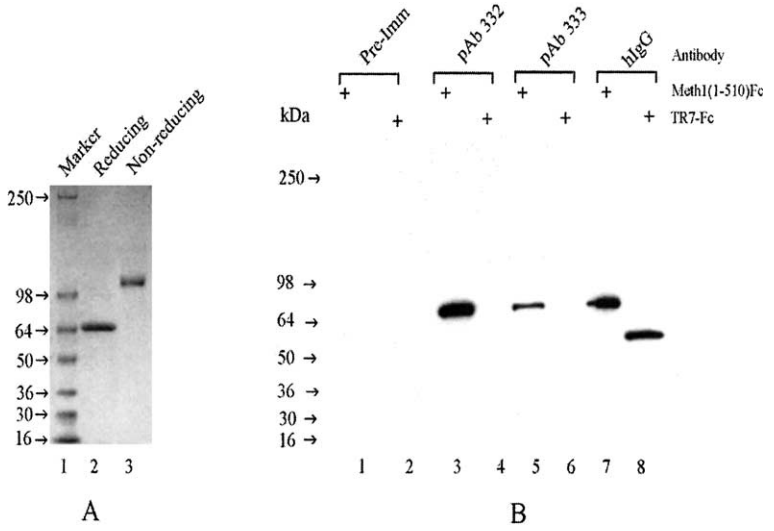

Fig. 5. Characterization of pC4Fc: METH-1 (residues 1–510) using different Abs. The METH-1 (residues 1–510)-Fc protein was prepared by a 293T cell transient expression system and purified as described in Materials and methods. The purified METH-1 (residues 1–510)-Fc was separated by 10% SDS–PAGE and detected by Coomassie blue staining (A) and by immunoblotting using an ECL system (B). The purified METH-1 (residues 1–510) showed a single band (68 kDa) under reducing condition (A: lane 2) and a single band (105 kDa) under non-reducing condition (A: lane 3). The pC4Fc: METH-1 (residues 1–510) was added in B to lanes 1, 3, 5, and 7; TR7-Fc, a positive control for the Fc domain, was added to lanes 2, 4, 6, and 8. Blots were detected by immunoblotting using an ECL system by adding Pre-immuno-IgG of the same rabbit of anti-METH-1 metalloproteinase domain IgG (pAb332) (B: lanes 1 and 2), anti-METH-1 metalloproteinase domain IgG (pAb332) (B: lanes 3 and 4), anti-METH-1 cysteine-rich domain IgG (pAb333) (B: lanes 5 and 6), and anti-human IgG1 (Fc) Ab (B: lanes 7 and 8).

culture supernatant containing METH-1 proteins was mixed with an equal volume of α2-M solution. Using anti-human IgG1 Ab, we found that METH-1 (residues 1–614) (Fig. 6A, lanes 5 and 6; C, lanes 1 and 2) and METH-1 (residues 1–544) (Fig. 6B, lanes 1 and 2; C, lanes 3 and 4) formed complexes with α2-M. However, METH-1 (residues 1–510) (Fig. 6C, lanes 5 and 6), and METH-1 (residues 1–460) (Fig. 6B, lanes 5 and 6) did not form complexes with α2-M. These results were confirmed using an anti-metalloproteinase Ab (pAb332; data not shown). None of the E^{385} to A mutants formed complexes with α2-M (Fig. 6A, lanes 3, 4, 7, and 8; B, lanes 3 and 4). These results showed that the metalloproteinase domain alone is not sufficient to express enzymatic activity towards α2-M, thus, the minimum domain requirement for the enzymatic activity was investigated. Interestingly, α2-M and the full-length METH-1 (residues 1–950) complexes were not detected by the anti-Fc Ab (anti-human IgG1 Ab) (Fig. 6A, lanes 1 and 2). This is due to the C-terminal proteolytic removal of the 21 kDa Fc antigen epitope as demonstrated by the results shown in Fig. 3 and by protein N-terminal sequencing. As a result, the anti-IgG1 Ab does not recognize the protein bands any more. Using our specific polyclonal anti-METH-1 metalloproteinase domain peptide IgG (pAb332), we found that full-length METH-1 did form a complex with α2-M (Fig. 7, lanes 1 and 2). On the other hand, there is no complex formation with the E^{385} to A mutants of full-length METH-1 (residues 1–950E/AFc) in the presence of α2-M (Fig. 7, lanes 3 and 4).

pFLAG-METH-1 (residues 236–614) protein expression and α2-M binding assay

To test the hypothesis that both the metalloproteinase domain and disintegrin domain of METH-1 are necessary for METH-1 activity, we created the pFLAG-METH-1 (residues 236–614) vector (Fig. 1B) and expressed it in a 293T cell expression system. The control vector was also expressed in this system. After being cultured for 3 days, the CM were collected, the cells were lysed, and the ECM components were collected. The collected samples were separated by a 10% SDS–PAGE and transferred onto a nitrocellulose membrane. The blot was probed with an anti-FLAG M2 monoclonal Ab (Eastman Kodak) and the pFLAG-METH-1 (residues 236–614) bands were detected by immunoblotting using an ECL system. The α2-M binding assay using CM showed that the pFLAG-METH-1 (residues 236–614) could form three different molecular weight complexes (Complexes I, II, and III) (Fig. 8A, lane 4). The reason why three complexes were detected for this particular construct is not clear. One of the possibilities is the NH_2-terminal FLAG tag of the pFLAG-METH-1, which allows anti-M2 Ab to recognize all the active forms of METH-1 that may have COOH-terminal truncations and cannot be recognized by anti-FC IgG1 Ab. In addition, heterodimers or oligomers may be formed among subunits of α2-M and different active forms of METH-1. The vector control did not show a binding complex (Fig. 8A, lanes 1 and 2). Most of the pFLAG-METH-1 (residues 236–614) was found in the

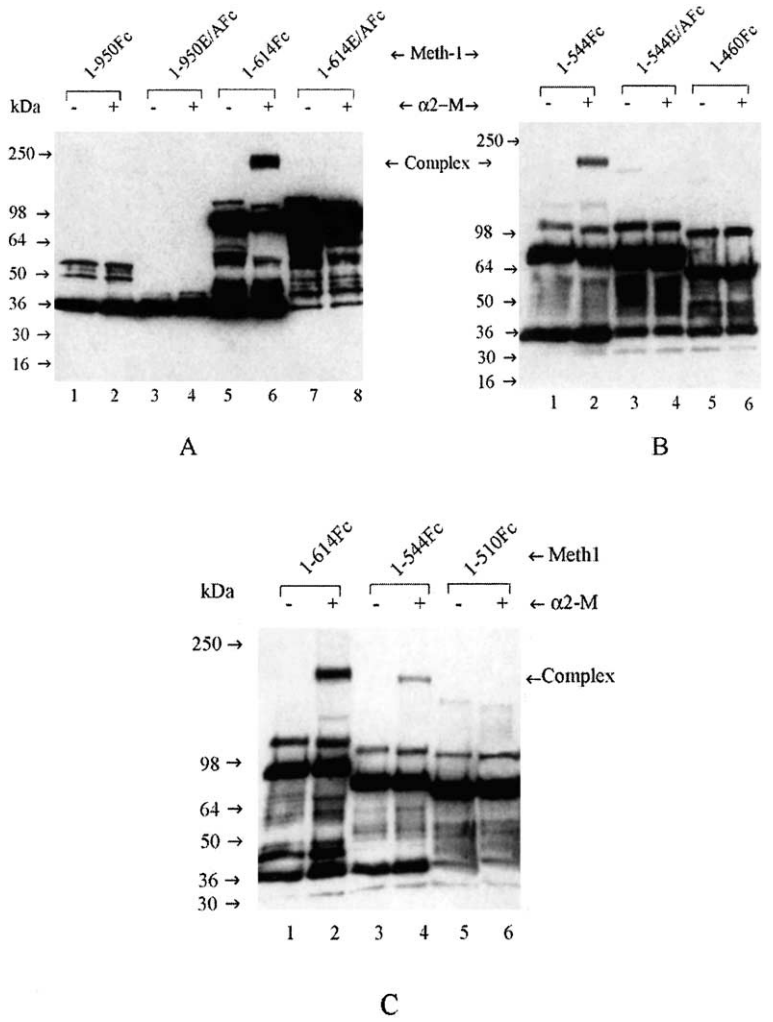

Fig. 6. Detection of high molecular weight complexes between the METH-1-Fc proteins and α2-M in the 293T cell expression system. The recombinant METH-1-Fc proteins were probed with 2.5 μg/ml anti-human IgG1 Ab and detected by immunoblotting using an ECL system. The high molecular weight complexes are indicated by arrows. The complexes of the wild type full-length METH-1 and α2-M were not detected by anti-human IgG1 Ab (A: lanes 1 and 2). The high molecular weight complexes were detected in recombinant METH-1:residues 1–614 A: lanes 5 and 6; C: lanes 1 and 2), residues 1–544 (B: lanes 1 and 2; C: lanes 3 and 4) in the presence of α2-M, but the wild type METH-1:residues 1–510 (C: lanes 5 and 6) and residues 1–460 (B: lanes 5 and 6) did not show any complex formation. The mutant METH-1:residues 1–950E/A (A: lanes 3 and 4), residues 1–614E/A (A: lanes 7 and 8), and residues 1–544E/A (B: lanes 3 and 4) eliminated the ability to bind to α2-M.

cell lysate (Fig. 8B, lane 2) and the least was in the ECM phase (Fig. 8B, lane 4). This might be due to pFLAG-METH-1 (residues 236–614) containing only a small part of the ECM-binding domains (Fig. 1).

Discussion

In this study, we demonstrated that the recombinant METH-1/ADAMTS1 proteins (full-length METH-1: residues 1–950, METH-1: residues 1–614, 1–544, and 236–614) with both metalloproteinase and disintegrin/cysteine-rich domains are capable of forming covalent complexes with α2-M. α2-M is a high-affinity proteinase inhibitor found abundantly in plasma and interstitial fluids. It is able to bind to various types of proteinases including serine proteinases, cysteine proteinases, and metalloproteinases [25–28]. Due to the complex formation of proteinases with α2-M being dependent on their proteolytic activities against the bait region [27], this assay is a useful tool for identifying the endopeptidase activity of the proteinases. The mechanism for the binding of proteinases to α2-M is the so-called "trapping mechanism". When the bait region in the middle of α2-M is cleaved by a proteinase, a conformational change in the α2-M subunits is triggered, leading to an encapsulation of the proteinase and subsequent covalent cross-linking between α2-M and the active proteinase. Loechel et al. [28] reported that human ADAM12 (meltrin α) is an active metalloproteinase by means of

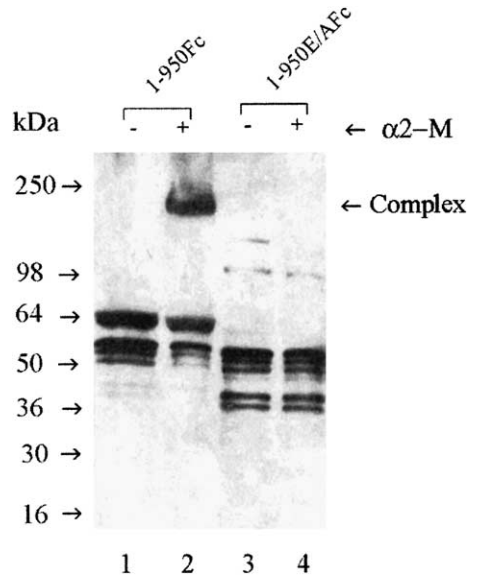

Fig. 7. Detection of high molecular weight complex of the full-length METH-1-Fc proteins and α2-M in the 293 T cell expression system. The recombinant METH-1-Fc proteins from the full-length (residues 1–950) construct were probed with 1 μg/ml anti-METH-1 metalloproteinase domain IgG (pAb332) and detected by immunoblotting using an ECL system. The high molecular weight complex is seen in lane 2, slightly below 250 kDa.

this trapping mechanism of α2-M. Mouse ADAMTS1 also had active proteinase activity based on its ability to form a covalent complex with α2-M [16].

The METH-1 (residues 1–510, 1–460) proteins lacking disintegrin/cysteine-rich domain could not form complexes with α2-M. These results indicated that the METH-1 metalloproteinase domain is only active in the presence of the intact disintegrin/cysteine-rich domain. Therefore, the whole disintegrin/cysteine-rich domain of METH-1 is required for METH-1 proteolytic activity with α2-M. The present data supply direct evidence for the hypothesis that the substrate interacts with both the metalloproteinase and disintegrin/cysteine-rich domains of METH-1, which was predicted by Black and White for other ADAMs [29]. In addition, all of the point mutants in the zinc-binding motif (E^{385} to A) of METH-1 proteins (METH-1: residues 1–950E/A, 1–614E/A, 1–544E/A, 1–510E/A) had no catalytic activity and did not form complexes with α2-M. Thus, introduction of a point mutation in the zinc-binding site (Glu^{385} to Ala) abolished the catalytic activity, demonstrating that Glu^{385} is probably a catalytic residue that plays an essential role in enzymatic reaction.

Sequence analysis of METH-1 predicted that the enzyme was synthesized as a zymogen that might be processed by furin in transit through the secretory pathway; a basic motif ($R^{232}KKR^{235}$) was found between the propeptide domain and the metalloproteinase domain. Furin is a serine endopeptidase in the trans-Golgi network (TGN) and is expressed in a variety of cells. It cleaves a wide range of precursor proteins including MDC15 and ADAMTS1 [16,30]. In the present

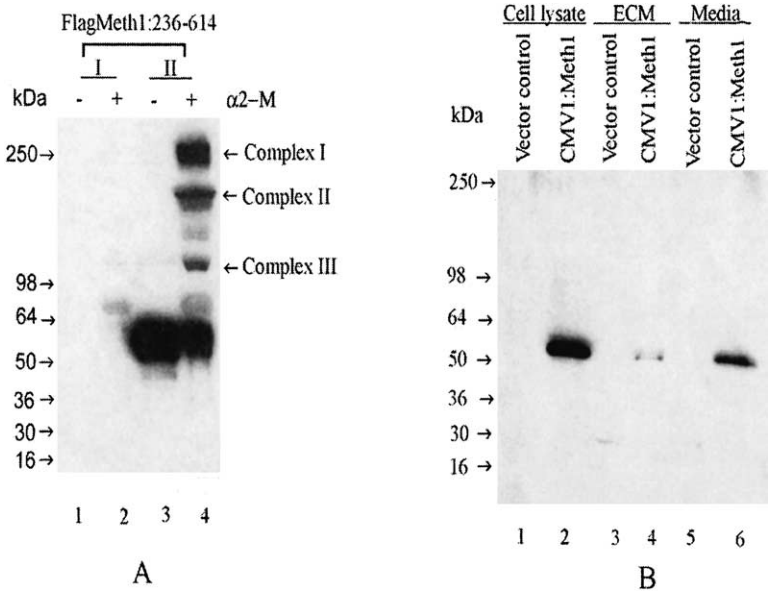

Fig. 8. α2-M Trapping assay and distribution of pFLAG: METH-1 (residues 236–614). A: High molecular weight bands of pFLAG: METH-1 (residues 236–614) were detected in the 293T cell expression system by immunoblotting using an ECL system. Phase I is the CM of the vector as a control (A: lanes 1 and 2). Phase II is the pFLAG:METH-1 (residues 236–614) transfected 293T cells CM (A: lanes 3 and 4). The high molecular weight complexes are indicated by arrows. B: Lanes 1 and 2 are cell lysate phases; lanes 3 and 4 are ECM phases; lanes 5 and 6 are CM. Lanes 1, 3, and 5 are CMV1 vector control and lanes 2, 4, and 6 are pFLAG-CMV1:METH-1 (residues 236–614). The pFLAG:METH-1 (residues 236–614) were found mainly in the cell lysate phase (B: lanes 1 and 2). The CM also contained a moderate amoung of pFLAG:METH-1 (residues 236–614) (B: lanes 5 and 6). The least amount of pFLAG: METH-1 (residues 236–614) was found in the ECM phase (B: lanes 3 and 4).

study, we show that the amino-terminal sequence of the major bands of all the recombinant METH-1 proteins is F^{236}VSSHRYV and it is located just after this proprotein convertase recognition site R^{232}KKR^{235}–F^{236}. Even the catalytically inactive mutants of METH-1 are proteolytically processed at this site. These data correlate well with the hypothesis that the propeptide domain of the METH-1 protein is removed in the secretory pathway reported by Kuno et al. [16] using the furin-deficient LoVo cells. More recently, Rodriguez-Manzaneque et al. [15] demonstrated that METH-1 could be processed in two consecutive steps to release active forms of both p87 and p65. The p87 fragment was generated by furin, while the p65 was generated by matrix metalloproteinases (MMP-2, -8, and -15).

A sequence comparison of the active ADAM proteases with the snake venom metalloproteinases reveals a consensus sequence for the zinc-binding motif, HEXGHXXGXXHD. Furthermore, from analyses of a number of zinc endopeptidases, including the snake venom metalloproteinases, astacin, seralysins, and matrix metalloproteinases (MMPs), it appears that the zinc-binding motif has a more general sequence, HEXXHXXGXXH [31,32]. However, in the potential zinc-binding motif (HEXXHXXGXXH) of METH-1 protein, the Gly (G) residue is replaced by Asn (N^{391}). The methionine residue (M^{412}) may function as a Met turn residue, as shown in MMPs. Since the present studies have demonstrated that METH-1 (residues 1–614, 1–544)-Fc and pFLAG-METH-1 (residues 236–614) are active as revealed by the α2-M binding assays, it is possible that the conserved G residue of the zinc-binding motif is functionally interchangeable with N. Kuno et al. found that ADAMTS1 cleaved the Glu^{1871}–Leu^{1872} bond within the chondroitin sulfate attachment domain of aggrecan [17]. Human aorta versican, a large aggregating proteoglycan, was cleaved at the Glu^{441}–Ala^{442} bond by ADAMTS1 [33]. Those results suggest that ADAMTS1 may be involved in the turnover of aggrecan and other types of proteoglycans in vivo.

ADAMTS1 is associated with the ECM through multiple ECM-binding domains (three TSP1 type 1 repeats and the spacer region) (Figs. 1 and 9) in its carboxyl-terminal region, whereas its metalloproteinase domain is free, i.e., not bound to the ECM [16]. The present study has demonstrated that the pFLAG-METH-1 (residues 236–614), which lacks most of the ECM-binding domains, was mostly localized in the cell lysate and very little was found in the ECM phase. The recombinant METH-1 protein (METH-1: residues 1–614-Fc) with only the first TSP1 type 1 repeat and 16 amino residues (residues 599–614) of the spacer region and three other recombinant METH-1 proteins (METH-1: residues 1–544-Fc, 1–510-Fc, and 1–460-Fc) without the multiple ECM-binding domains were secreted into the cell CM. These results suggested that the ECM-binding domains of METH-1 proteins might anchor to the extracellular matrix after secretion.

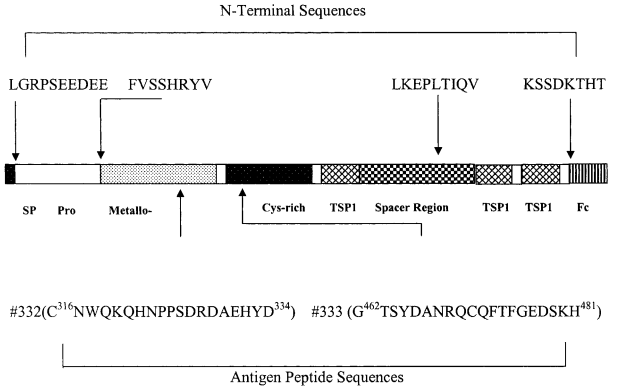

Fig. 9. Summary of N-terminal sequences of the processed forms and antigen peptide sequences in the full-length human METH-1/ADAMTS1-Fc. The domain labels are: SP, signal peptide; Pro, propeptide domain; metallo-, metalloproteinase domain; Cys-rich, disintegrin/cysteine-rich domains; TSP1, thrombospondin type-1 motif; Fc, immunoglobulin G constant fragment (COOH-terminal region). The experimentally determined N-terminal sequences of the processed forms are shown above the domain structure of the METH-1. The antigen peptide sequences used to make pAb332 and pAb333 are shown below the domain structure. The arrows point to the location of each peptide sequence in the domain structure.

More recently, using an ADAMTS1-null mice model, Shindo et al. [14] showed that targeted disruption of the mouse ADAMTS1 gene resulted in growth retardation with adipose tissue malformation and impaired female fertilization and histological changes in the uterus and ovaries. ADAMTS1 (−/−) mice had enlarged renal calices with fibrotic changes from the ureteropelvic junction through the ureter and abnormal adrenal medullary architecture without capillary formation. ADAMTS1 plays a significant role in the proteolytic and inflammatory events of the ovulation process [18,19,34]. Furthermore, ADAMTS1 demonstrates a structural similarity to GON-1, a metalloproteinase that controls gonadogenesis by remodeling the basement membrane in *Caenorhabditis elegans* [20]. Kuno et al. demonstrated that ADAMTS1 might play a role in the inflammatory process through its protease activity [11,13,16,35]. Diamantis et al. [36] reported that regulation of the expression of the ADAMTS1 gene might be important in the development of cirrhosis of liver in rat liver endothelial cells. METH-1 also participated in angiogenesis [10,37] and flow-dependent vascular adaptation [38].

Our present results show that METH-1 (residues 1–510), with the metalloproteinase domain and partial disintegrin/cysteine-rich domain, and METH-1 (residues 1–460)-Fc with only metalloproteinase domain are not active based on binding with α2-M. However, METH-1 (residues 1–950, 1–614, 1–544)-Fc and pFLAG-METH-1 (residues 236–614), with complete metalloproteinase

and disintegrin/cysteine-rich domains, show activity. This indicates that both the metalloproteinase domain and disintegrin/cysteine-rich domains may play important roles in physiological processes, including normal growth, fertility, organ morphology, and function, and ovulation; and pathological processes, including inflammation, liver cirrhosis, angiogenesis, and vascular adaptation.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health CA78646, the American Cancer Society, Florida Division F01FSU-1, and the Florida State University Research Foundation (to Q.-X.A.S.).

References

[1] T.G. Wolfsberg, P. Primakoff, D.G. Myles, J.M. White, ADAM, a novel family of membrane proteins containing a disintegrin and metalloprotease domain: multipotential functions in cell–cell and cell–matrix interactions, J. Cell Biol. 131 (1995) 275–278.

[2] C.P. Blobel, Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNF alpha and Notch, Cell 90 (1997) 589–592.

[3] C.P. Blobel, T.G. Wolfsberg, C.W. Turck, D.G. Myles, P. Primakoff, J.M. White, A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion, Nature 356 (1992) 248–252.

[4] I. Abbaszade, R.Q. Liu, F. Yang, S.A. Rosenfeld, O.H. Ross, J.R. Link, D.M. Ellis, M.D. Tortorella, M.A. Pratta, J.M. Hollis, R. Wynn, J.L. Duke, H.J. George, M.C. Hillman Jr., K. Murphy, B.H. Wiswall, R.A. Copeland, C.P. Decicco, R. Bruckner, H. Nagase, Y. Itoh, R.C. Newton, R.L. Magolda, J.M. Trzaskos, G.F. Hollis, E.C. Arner, T.C. Burn, Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family, J. Biol. Chem. 274 (1999) 23443–23450.

[5] A.L. Stone, M. Kroeger, Q.X. Sang, Structure–function analysis of the ADAM family of disintegrin-like and metalloproteinase-containing proteins (review), J. Protein Chem. 18 (1999) 447–465.

[6] G. Weskamp, J. Krätzschmar, M. Reid, C.P. Blobel, MDC9, a widely expressed cellular disintegrin containing cytoplasmic SH3 ligand domains, J. Cell Biol. 132 (1996) 717–726.

[7] T.G. Wolfsberg, J.M. White, ADAMs in fertilization and development, Dev. Biol. 180 (1996) 389–401.

[8] K. Iba, R. Albrechtsen, B.J. Gilpin, F. Loechel, U.M. Wewer, Cysteine-rich domain of human ADAM 12 (meltrin alpha) supports tumor cell adhesion, Am. J. Pathol. 154 (1999) 1489–1501.

[9] E. Abe, H. Mocharla, T. Yamate, Y. Taguchi, S.C. Manolagas, Meltrin-alpha, a fusion protein involved in multinucleated giant cell and osteoclast formation, Calcif. Tissue Int. 64 (1999) 508–515.

[10] F. Vazquez, G. Hastings, M.A. Ortega, T.F. Lane, S. Oikemus, M. Lombardo, M.L. Iruela-Arispe, METH-1, a human ortholog of ADAMTS1, and METH-2 are members of a new family of proteins with angio-inhibitory activity, J. Biol. Chem. 274 (1999) 23349–23357.

[11] K. Kuno, N. Kanada, E. Nakashima, F. Fujiki, F. Ichimura, K. Matsushima, Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene, J. Biol. Chem. 272 (1997) 556–562.

[12] P. Bornstein, Thrombospondins: structure and regulation of expression, FASEB J. 6 (1992) 3290–3299.

[13] K. Kuno, K. Matsushima, ADAMTS1 protein anchors at the extracellular matrix through the thrombospondin type I motifs and its spacing region, J. Biol. Chem. 273 (1998) 13912–13917.

[14] T. Shindo, H. Kurihara, K. Kuno, H. Yokoyama, T. Wada, Y. Kurihara, T. Imai, Y. Wang, M. Ogata, H. Nishimatsu, N. Moriyama, Y. Oh-hashi, H. Morita, T. Ishikawa, R. Nagai, Y. Yazaki, K. Matsushima, ADAMTS1: a metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function, J. Clin. Invest. 105 (2000) 1345–1352.

[15] J.C. Rodriguez-Manzaneque, A.B. Milchanowski, E.K. Dufour, R. Leduc, M.L. Iruela-Arispe, Characterization of METH-1/ADAMTS1 processing reveals two distinct active forms, J. Biol. Chem. 275 (2000) 33471–33479.

[16] K. Kuno, Y. Terashima, K. Matsushima, ADAMTS1 is an active metalloproteinase associated with the extracellular matrix, J. Biol. Chem. 274 (1999) 18821–18826.

[17] K. Kuno, Y. Okada, H. Kawashima, H. Nakamura, M. Miyasaka, H. Ohno, K. Matsushima, ADAMTS1 cleaves a cartilage proteoglycan, aggrecan, FEBS. Lett. 478 (2000) 241–245.

[18] R.L. Robker, D.L. Russell, L.L. Espey, J.P. Lydon, B.W. O'Malley, J.S. Richards, Progesterone-regulated genes in the ovulation process: ADAMTS1 and cathepsin L proteases, Proc. Natl. Acad. Sci. USA 97 (2000) 4689–4694.

[19] L.L. Espey, S. Yoshioka, D.L. Russell, R.L. Robker, S. Fujii, J.S. Richards, Ovarian expression of a disintegrin and metalloproteinase with thrombospondin motifs during ovulation in the gonadotropin-primed immature rat, Biol. Reprod. 62 (2000) 1090–1095.

[20] R. Blelloch, J. Kimble, Control of organ shape by a secreted metalloprotease in the nematode Caenorhabditis elegans, Nature 399 (1999) 586–590.

[21] P. Wei, Y.G. Zhao, L. Zhuang, S. Ruben, Q.X. Sang, Expression and enzymatic activity of human disintegrin and metalloproteinase ADAM19/meltrin beta, Biochem. Biophys. Res. Commun. 280 (2001) 744–755.

[22] Q.X. Sang, H. Birkedal-Hansen, H.E. Van Wart, Proteolytic and non-proteolytic activation of human neutrophil progelatinase B, Biochim. Biophys. Acta 1251 (1995) 99–108.

[23] H. Li, D.E. Bauzon, X. Xu, H. Tschesche, J. Cao, Q.A. Sang, Immunological characterization of cell–surface and soluble forms of membrane type 1 matrix metalloproteinase in human breast cancer cells and in fibroblasts, Mol. Carcinog. 22 (1998) 84–94.

[24] D.R. Hurst, H. Li, X. Xu, V.L. Badisa, Y.E. Shi, Q.X. Sang, Development and characterization of a new polyclonal antibody specifically against tissue inhibitor of metalloproteinases 4 in human breast cancer, Biochem. Biophys. Res. Commun. 281 (2001) 166–171.

[25] R.D. Feinman, The proteinase-binding reaction of alpha 2M, Ann. N. Y. Acad. Sci. 737 (1994) 245–266.

[26] L. Sottrup-Jensen, Role of internal thiol esters in the alpha-macroglobulin-proteinase binding mechanism, Ann. N. Y. Acad. Sci. 737 (1994) 172–187.

[27] H. Nagase, Y. Itoh, S. Binner, Interaction of α2-macroglobulin with matrix metalloproteinases and its use for identification of their active forms, Ann. N. Y. Acad. Sci. 732 (1994) 294–302.

[28] F. Loechel, B.J. Gilpin, E. Engvall, R. Albrechtsen, U.M. Wewer, Human ADAM 12 (meltrin alpha) is an active metalloprotease, J. Biol. Chem. 273 (1998) 16993–16997.

[29] R.A. Black, J.M. White, ADAMs: focus on the protease domain, Curr. Opin. Cell Biol. 10 (1998) 654–659.

[30] J.B. Bjarnason, J.W. Fox, Snake venom metalloendopeptidases, reprolysins, Methods Enzymol. 248 (1994) 345–368.

[31] G.J.P. Murphy, G. Murphy, J.J. Reynolds, The origin of matrix metalloproteinases and their familial relationships, FEBS Lett. 289 (1991) 4–7.

[32] N.D. Rawlings, A.J. Barrett, Evolutionary families of metallopeptidases, Methods Enzymol. 248 (1995) 183–228.
[33] J.D. Sandy, J. Westling, R.D. Kenagy, M.L. Iruela-Arispe, C. Verscharen, J.C. Rodriguez-Mazaneque, D.R. Zimmermann, J.M. Lemire, J.W. Fischer, T.N. Wight, A.W. Clowes, Versican V1 proteolysis in human aorta in vivo occurs at the Glu441–Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4, J. Biol. Chem. 276 (2001) 13372–13378.
[34] R.L. Robker, D.L. Russell, S. Yoshioka, S.C. Sharma, J.P. Lydon, B.W. O'Malley, L.L. Espey, J.S. Richards, Ovulation: a multi-gene, multi-step process, Steroids 65 (2000) 559–570.
[35] K. Kuno, H. Lizasa, S. Ohno, K. Matsushima, The exon/intron organization and chromosomal mapping of the mouse ADAMTS1 gene encoding an ADAM family protein with TSP motifs, Genomics 46 (1997) 466–471.
[36] I. Diamantis, M. Luthi, M. Hosli, J. Reichen, Cloning of the rat ADAMTS1 gene and its down regulation in endothelial cells in cirrhotic rats, Liver 20 (2000) 165–172.
[37] M.L. Iruela-Arispe, F. Vazquez, M.A. Ortega, Antiangiogenic domains shared by thrombospondins and metallospondins, a new family of angiogenic inhibitors, Ann. N. Y. Acad. Sci. 886 (1999) 58–66.
[38] M. Bongrazio, C. Baumann, A. Zakrzewicz, A.R. Pries, P. Gaehtgens, Evidence for modulation of genes involved in vascular adaptation by prolonged exposure of endothelial cells to shear stress, Cardiovasc. Res. 47 (2000) 384–393.