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## Identification of a thrombin cleavage site and a short form of ADAMTS-18

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### Abstract

We previously reported that C-terminal fragment of ADAMTS-18 induces platelet fragmentation through ROS release. We have shown that thrombin cleaves ADAMTS-18 and that a short form of ADAMTS-18 in *in vitro* translational assay. However, the exact thrombin cleavage site and whether a short form ADAMTS-18 presents in vivo are not clear. In this study, we first identified that the thrombin cleavage site is between Arg775 and Ser776 by thrombin cleavage of ADAMTS-18 peptide following mass spectrum assay. We then showed that a short form ADAMTS-18 presents in brain, kidney, lung, and testicle from C57BL/6 mouse embryo. Since alternative form of ADAMTS-18 could be a mechanism to regulate its activity, we then investigated the mechanism involves in the generation of ADAMTS-18 short form. However, neither protease inhibitors nor mutations in catalytic domain of ADAMTS-18 have any significant effect on the generation of ADAMTS-18 short form. Thus, our data demonstrate a thrombin cleavage site and confirm a short form of ADAMTS-18 presents in vivo.

### Keywords

Metalloproteinase; Thrombin; Cleavage; ADAMTS-18

### Introduction

The human ADAMTSs (a disintegrin and metalloproteinase with a thrombospondin type 1 motif) are a family of secreted Zn-metalloproteinases, which have multidomain structural components in common.[1; 2; 3; 4] The functions of members of the ADAMTS family include N-terminal procollagen processing (ADAMTS-2, -3, -14),[5; 6; 7; 8] spermatogenesis (ADAMTS-2),[9] inhibition of angiogenesis (ADAMTS-1, -8, and -9),[10; 11] follicular rupture and ovulation (ADAMTS- 1),[12] cleavage of matrix proteoglycans aggrecan, versican, and brevican (ADAMTS-1, -4, -5, -8, -9, -15),[13; 14; 15] degradation of cartilage oligomeric matrix protein (ADAMTS-7, ADAMTS-12), and cleavage of ultra large molecular weight von Willebrand factor (ADAMTS-13).[3] ADAMTS-18 has recently been shown to be epigenetically silenced in multiple carcinomas and to have tumor suppressor activity.[16] We have shown that C-terminal fragment of ADAMTS-18 induces platelet fragmentation through ROS (reactive oxygen species).[1] Although we reported that

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thrombin cleaves ADAMTS-18, the exact thrombin cleavage site and how the activity of ADAMTS-18 being regulated are still unknown.

The regulation of metalloprotease activity could be at three levels: transcriptional regulation, zymogen activation, and regulation on the level of enzymatic activity by different endogenous regulators such as protease cleavage or inhibitors.[17; 18] At the transcriptional level, it has been shown that ADAMTS-16 expression is stimulated by TGF $\beta$  in chondrocyte cell lines and by follicle-stimulating hormone (FSH) in fully differentiated luteinizing granulosa cells.[19; 20] The mRNA level of ADAMTS-8 is down-regulated in brain tumor and TNF $\beta$  is able to up-regulate ADAMTS-18 mRNA level in endothelial cells.[1; 21; 22] The ADAMTSs activity can also be regulated by proteolytic process.[23] All known ADAMTSs (except 10 and 12) contain a subtilisin-like pro-protein convertase cleavage site in their prodomains that are furin recognition sequences. ADAMTS can be cleaved at the N-terminal by furin or related pro-protein convertase(s) within the trans-Golgi, resulting in secretion of mature, potentially active enzymes lacking the propeptide region.[1; 3] In addition, ADAMTS family members such as ADAMTS-1 and ADAMTS-12 have been shown to undergo proteolytic processing within their C-terminal regions, resulting in removal of domains that can bind to sulfated GAGs.[9; 24] It has been shown that C-terminal truncation enhances the aggrecanase and versicanase activities of ADAMTS-4, indicating a potential regulatory function associated with one or more domains of the ADAMTS-4 C-terminal region. [25; 26]

Alteration of ADAMTSs activity has been implicated with certain physiological conditions *in vivo*. It has been shown that following transient middle cerebral artery occlusion in the rat, ADAMTS-1 and -4 are up-regulated.[27] An orderly temporal expression of the metalloproteinases and ADAMTS has been shown during the progression of fracture healing.[28] We have reported that thrombin cleaves ADAMTS-18 and releases C-terminal fragment and shown that a short form of ADAMTS-18 was also present during *in vitro* translation of full length ADAMTS-18.[22] However, the exact thrombin cleavage site and whether the short form presents *in vivo* are not clear. Thus, to better understand the function of ADAMTS-18, we have investigated the thrombin cleavage site and the expression of short form ADAMTS-18 *in vivo*.

## Materials and Methods

### Reagents and plasmid

All reagents were purchased from Sigma unless otherwise designed. ADAMTS-18 peptide was synthesized by Bio-Synthesis (Lewisville, TX). The *in vitro* translation kit was purchased from Promega (Madison, WI USA). Full-length ADAMTS-18 cDNA coding sequence was purchased from ATCC (Manassas, VA ) and cloned into mammalian expression vector pBudCE4.1 from Invitrogen (Carlsbad, CA). pCR3.1/ADAMTS-18 was kindly provided by Dr. Andrew Connolly (Stanford University, CA). Optimized ADAMTS-18-cDNA was synthesized by GenScript (Piscataway, NJ) and then cloned into pcDNA3.1. Protease inhibitors Complete Mini Cocktail and Complete Mini EDTA-free were purchased from Roche (Mannheim, Germany).

### Peptide synthesized and mass spectrum assay

ADAMTS-18 peptide was digested with thrombin (5 U/ml) at room temperature for one hour with/without huridin (5 ug/ml). The digested samples were analyzed by mass spectrum assay at NYULMC protein core facility. Briefly, 10 mg/ml Alpha-Cyano-4-Hydroxycinnamic Acid (CHCA, Agilent Technologies) was used as the MALDI matrix. A total of 1.0  $\mu$ L of sample in 50% acetonitrile and 0.1% TFA was mixed with equal volume

of matrix solution, spotted on the MALDI plate, and air-dried. Samples for mass spectrometry determination were analyzed in positive, reflectron mode on a Bruker Autoflex (Billerica, MA). Samples for peptide sequence determination were analyzed in positive, reflectron mode on a Waters Q-ToF Ultima (Milford, MA).

### **In vitro translation**

<sup>35</sup>S-methionine-labeled ADAMTS-18 was translated from pBudCE4.1/AD18, pBudCE4.1/AD18 mutants, pBluescript/AD18, pcDNA3.1/AD18, or pCR3.1/AD18 using an in vitro translation kit (TNT Coupled Reticulocyte Lysate Systems; Promega, Madison, WI) following the protocol provided by the manufacturer. Various protease inhibitors: Complete Mini Cocktail, Complete Mini(-EDTA) 100ug/ml or  $\alpha$ 2-macroglobulin (4mU-16mU) were added to the reactions. The translational products were resolved on SDS-PAGE gel. Film was developed by exposing to the dried gel at 4°C overnight.

### **Construct the catalytic domain mutant ADAMTS-18 expression vector**

Mutant primers were synthesized by Sigma. The point mutations were introduced into the catalytic domain of ADAMTS-18 as previous described.[29; 30] Briefly, 10ng DNA fragments from the ADAMTS-18 plasmid were amplified using high fidelity Pfu from Stratagene (Santa Clara, CA) in a final volume of 100 u1 and subjected to 30 cycles of denaturation (1 min, 94 °C), annealing (2 min, 50 °C), and extension (3 min, 72 °C) using a DNA Thermal Cycler (Perkin Elmer, Waltham, MA). The products of the reaction were analyzed and purified through agarose gel electrophoresis. Purified DNA fragments from an initial set of reactions were used in a subsequent overlap extension reaction. Overlapping fragments were mixed and subjected to PCR amplification using the external primers. Full length ADAMTS-18 mutant fragment was purified from agarose gel using Qiagen PCR product purification kit and then cloned into pcDNA3.1. Sequences of all constructs were confirmed by sequencing.

### **Immunoblotting**

Twenty-five micrograms of protein from a variety of tissue lysis from C57BL/6 mice embryo were separated by 7% SDS/PAGE gels and transferred to a PVDF membrane before immunoblotting with rabbit anti-ADAMTS-18 IgG (sc-68416, Santa Cruz Biotechnology, Inc.) overnight (1:1000 dilution). The membrane was then blotted with Biotin conjugated anti-Rabbit IgG (1:10,000) for 2 h, followed by horseradish peroxidase (HRP)-conjugated Avidin (1:20,000) for half hour. The ADAMTS-18 band was detected with chemiluminescence substrate (Thermo Fisher Scientific, Rockford, IL).

## **Results**

### **Primary thrombin cleavage site is between Arg775 and Ser776**

Previously, we have reported that thrombin is able to cleave ADAMTS-18 and release a C-terminal fragment.[22] To identify the thrombin cleavage site, we have synthesized a ADAMTS-18 peptide containing the predicted putative thrombin cleavage site (Figure 1a,b). Mass spectrum assay of ADAMTS-18 peptide cleaved by thrombin reveals two peptide peaks (MW1561 and MW1447), which reflect molecular weight of half the size of the synthesized peptide (Figure 1c). Mass spectrum sequencing confirms that MW1561 is the N terminal end of the peptide suggesting that the thrombin cleavage site is between Arg775 and Ser776. Hirudin completely inhibited the generation of MW1561 and MW1447 peak suggesting the specificity of thrombin cleavage (Figure 1c).

### **In vitro translation of ADAMTS-18 shows a short form**

<sup>35</sup>S-methionine-labeled ADAMTS-18 was generated by in vitro translation using the expression vector pBudCE4.1/ADAMTS-18. Since a non-specific band sometimes also presents in an in vitro translation assay, pBudCE4.1/ $\beta$ 3 integrin was used as a control. As shown in Figure 2a, pBudCE4.1/AD18 has two bands (135 kd and 75 kd). There is only one 85kd band in control. In addition, since 75 kd band was also found in translational product using other clone vectors: pBluescript/ADAMTS-18, pCR3.1/ADAMTS-18, our data suggests that the 75 kd band is indeed from ADAMTS-18 cDNA (Figure 2b).

### **Genetic codon optimization has no effect on production of short form ADAMTS-18**

ADAMTS-18 contains a significant amount of rare genetic codons. These rare codons may lead to the premature translational stop.[31] To test whether the formation of the short is due to such a rare genetic codon, we re-synthesized the full length cDNA of ADAMTS-18 with optimized genetic code and cloned into pcDNA3.1. Again, the optimization of ADAMTS-18 has no effect on the short form ADAMTS-18 formation (Figure 2b).

### **Short form of ADAMTS-18 exists in brain, kidney, lung, and testicle from C57BL/6 embryo**

To confirm whether the short form of ADAMTS-18 is present in vivo, we examined ADAMTS-18 expression in normal tissues by western blot. Protein samples from tissues of mouse embryo: brain, kidney, lung, and testicle were resolved on SDS-PAGE gel and transferred to PVDF membrane to detect ADAMTS-18. Our result shows that both two forms of ADAMTS-18 present in these tissues (Figure 2c).

### **Protease inhibitors and mutations in catalytic domain have no effect on 75 kd ADAMTS-18 formation**

We next explored the potential mechanism for the generation of the short form ADAMTS-18. To test whether the 75 kd form is due to protease cleavage, in vitro translation was done in the presence of a variety of protease inhibitor. Protease inhibitors have no significant effect on the generation of the short form (Figure 3a,b). Since ADAMTS-18 is the closest homology to ADAMTS-16, which has been shown to be able to cleave  $\alpha$ 2-macroglobulin, we decided to test whether the short form is due to the autocatalytic activity of ADAMTS-18.[20; 32] Point mutations were introduced into the catalytic domain based on previous reports (Figure 3c).[33; 34; 35; 36] These mutations in ADAMTS-18 catalytic domain had no effect on the generation of the 75 kd form in in vitro translation assays (Figure 3d). Thus, it appears another mechanism may be involved in the generation of the 75 kd ADAMTS-18.

## **Discussion**

Alternative forms have been reported in many ADAM members as well as ADAMTS members. Different isoforms resulting from alternative splicing have been shown in ADAM-8,-9,-10,-11,-12,-15,-19,-22,-28,-29,-30, and -33 or ADAMTS-4, ADAMTS-6.[23] Isoforms could also result from post-translational modulations from a processing of the metalloproteinase domain itself or by other metalloproteinases.[23] The different forms of ADAMTS could function differently in vivo. It has been shown that there are two distinct active forms of ADAMTS-1 generated by two consecutive steps to release p87 and p65 forms. The full length of ADAMTS-1 and ADAMTS-1 fragments possess pro- or anti-metastatic activity, respectively.[37; 38] Thus, it is of particular interest to identify whether a ADAMTS member has an isoform and what its function may be.

In this study, we have identified the thrombin cleavage site and confirmed the presence of a short form of ADAMTS-18 in vivo. Although the potential function of the short form is not

clear, our result has revealed a potential mechanism may involve in the regulation of ADAMTS-18 activity. The changes of ADAMTS-18 activity could be of significant biological implication in vivo. In fact, previous publications have shown that ADAMTS-18 expression level is associated with the tumor metastasis.[39] The expression profile of ADAMTS-18 has been studied in gastric, colorectal and pancreatic cancers and the a down regulation of ADAMTS-18 is associated with DNA methylation of its promoter.[40] The methylation levels in ADAMTS-18 gene promoter region has been studied in tissue from patients samples:100 gastric cancers, 100 colorectal cancers, 70 pancreatic cancers, and equal number of adjacent normal tissues. Methylation levels in all three types of cancers were significantly higher compared to normal tissues. This is the first study to estimate the prevalence of ADAMTS-18 methylation based on large amount of tumor samples, showing that epigenetic regulation of ADAMTS-18 expression level is associated with carcinogenesis.[40] Further studies are needed for better understand how ADAMTS-18 activity is regulated and what the biological effect could result.

Since the protease inhibitors used in this study do not inhibit the formation of short form, the potential mechanism underlying the generation of short form ADAMTS-18 is not clear. It is possible that the protease inhibitors do not completely inhibit all proteases activity given the fact that ADAMTS-16 the closest homology of ADAMTS18 could cleave  $\alpha 2$ -Macroglobulin.[20] Other possibilities could be either there is a cryptic translation start site or a translation stop site. It has been shown that RNA secondary structure can affect the translation.[41] Using computer program Mfold (Mfold Web Server, The RNA Institute, Albany, SUNY), we have found a similar mRNA secondary structure with mRNA sequences from wildtype ADAMTS-18 or codon optimized ADAMTS-18. In addition, it has been reported that the 5' untranslated region of ADAMTS-6 may be involved in translational control.[42] Thus, the role of mRNA secondary structure in generation of ADAMTS-18 short form cannot be excluded. Further study will help us understand better how ADAMTS-18 short form is generated in vivo.

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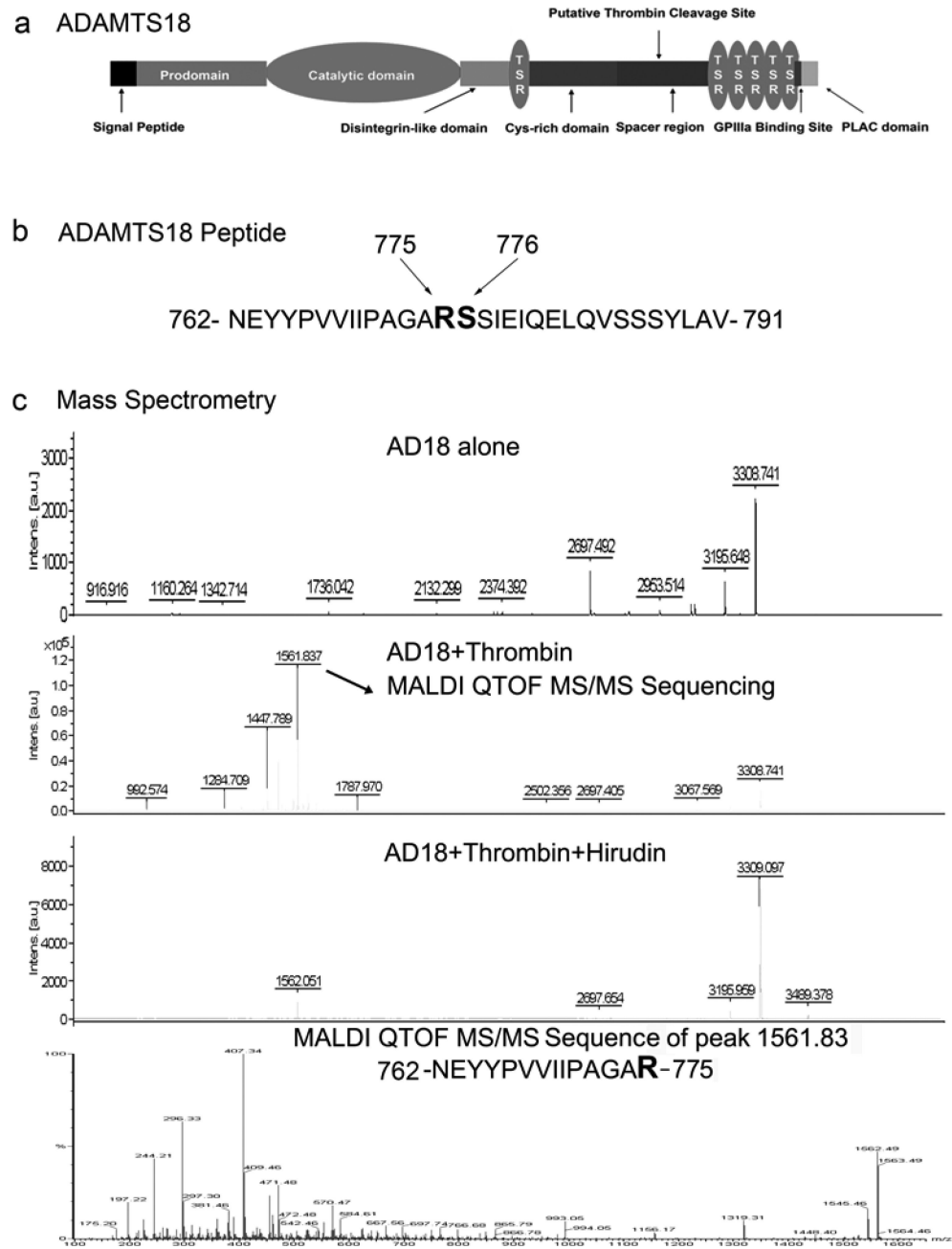
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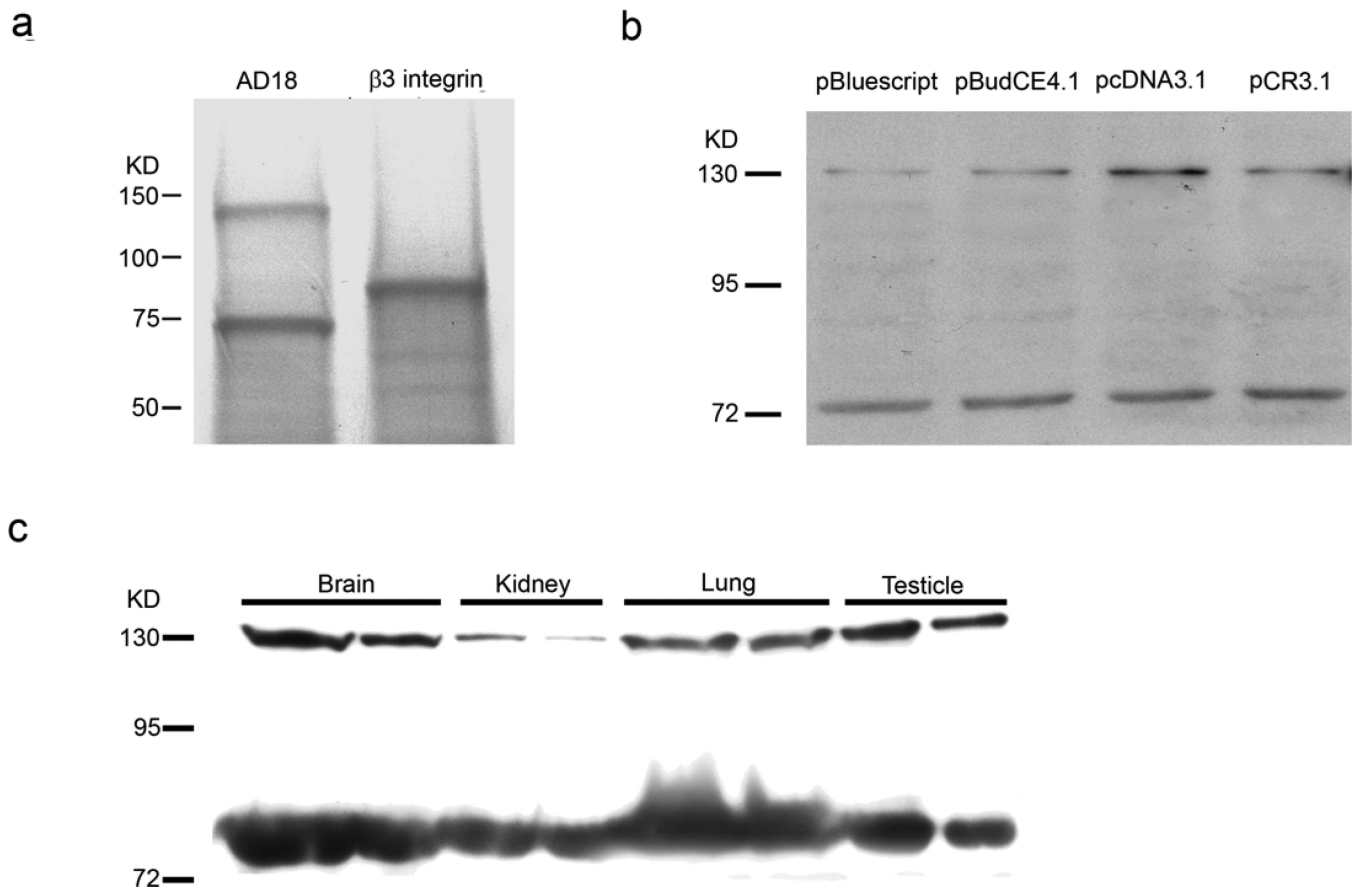


- Previously, we have shown that thrombin cleaves ADAMTS-18 and the cleavage site is not clear.
- In present study, we have identified that the thrombin cleavage site is between Arg775 and Ser776.
- Our data also reveals that a short form ADAMTS-18 presents in a variety of C57BL/6 tissues.



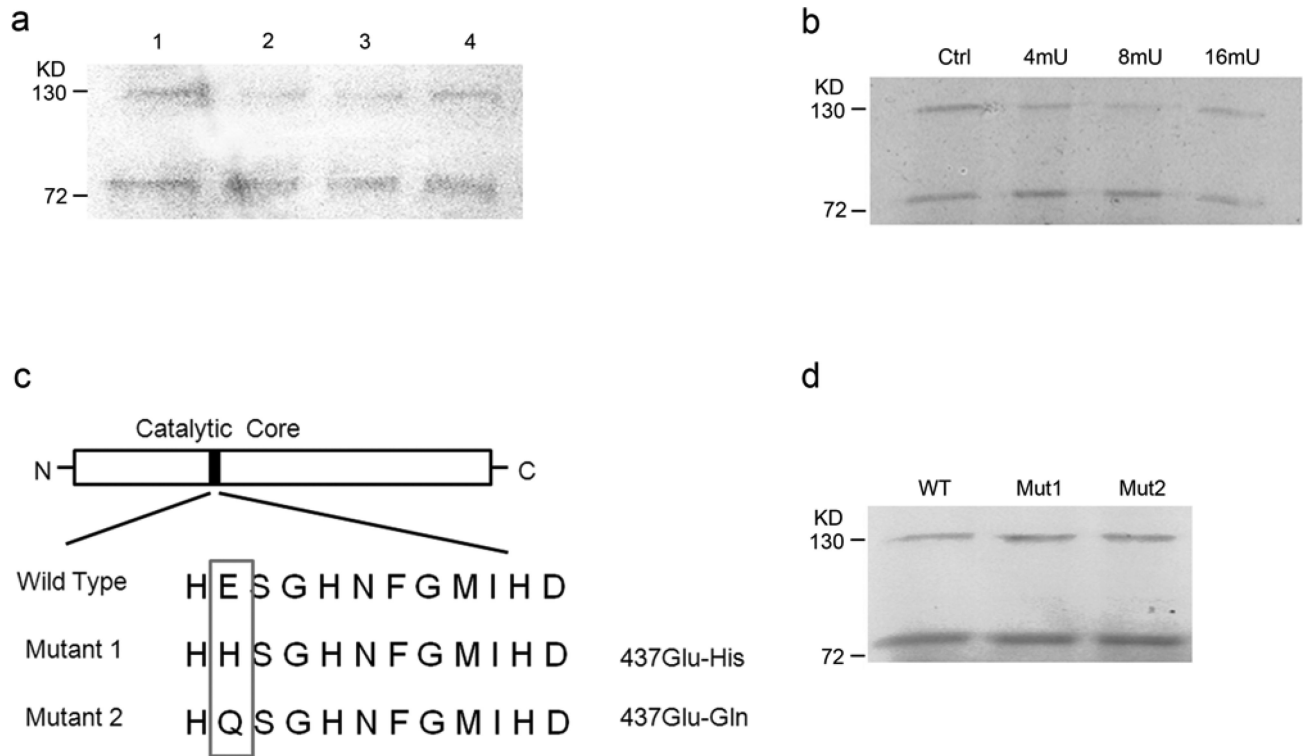
**Figure 1.**

a. Schematic of ADAMTS-18 showing putative thrombin cleavage site; b. Synthesized ADAMTS-18 peptide with predict thrombin cleavage site; c. Mass spectrometry assay to confirm thrombin cleavage site.



**Figure 2.**

a. In vitro translation using pBudCE4.1/ ADAMTS-18 and pBudCE4.1/ $\beta$ 3 integrin ; b. In vitro translation using pBluescript/ADAMTS-18, pBudCE4.1/ADAMTS-18, pcDNA3.1/ADAMTS-18 (genetic codon optimized cDNA), and pCR3.1/ADAMTS-18; c. Western blot to detect ADAMTS-18 in brain, kidney, lung, and testicle from C57BL/6 mouse embryo.

**Figure 3.**

a. In vitro translation of pBudCE4.1/ADAMTS-18 with protease inhibitors: Control (lane 1); Complete Mini EDTA free 100ug/ml (lane 2); Complete Mini Cocktail 100ug/ml (lane 3);  $\alpha$ 2-macroglobulin 4mU/ml (lane 4); b. In vitro translation of pBudCE4.1/ADAMTS-18 with  $\alpha$ 2-macroglobulin: Control (lane 1); 4mU(lane 2); 8mU(lane 3); 16mU(lane4); c. Schematic of point mutations introduced into ADAMTS-18 catalytic domain; d. In vitro translation of pBudCE4.1/ADAMTS-18 wildtype and mutants.