Arg-Ser-775, 792 and 823 in Spacer Region of ADAMTS-18 Is Critical for Thrombin Cleavage

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Abstract

Cleavage of ADAMTS-18 by thrombin represents a new mechanism of platelet thrombus clearance via the release of active ~45-kDa C-terminal fragments that induces oxidative platelet fragmentation. The exact cleavage sites remain unclear, but Arg (R)775/Ser (S)776 in spacer region of ADAMTS-18 has been shown to be one of the cleavage sites of thrombin. Here, we demonstrate that R792/S793 and R823/S824 are also thrombin cleavage sites by sequence analysis, amino acid mutation and mass spectrometry assay. All these cleavage sites are thrombin-specific and insensitive to other enzymes tested (e.g. cathepsin D or trypsin). Simultaneous mutation of R775, 792, 823 to S775, 792, 823 in ADAMTS-18 completely abrogated the cleavage by thrombin and the generation of active C-terminal 45-kDa fragments. Together with previous study, a total of three thrombin-specific cleavage sites have been identified in spacer region of ADAMTS-18.

Keywords

ADAMTS-18, Thrombin, Cleavage, Mass Spectrum

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1. Introduction

ADAMTS (a disintegrin and metalloproteinase domain, with thrombospondin type-1 modules) is a family of 19 secreted Zn-metalloproteinases, which have multidomain structural components in common [1]. These include an N-terminal signal peptide, followed by a pro-domain, a metalloproteinase catalytic domain with a zinc binding motif, a disintegrin-like domain, a central thrombospondin type-1-like repeat (TSR), a cysteine rich domain (high sequence homology), a spacer region, and a variable number of C terminal TSR repeats. This family plays important roles in several pathophysiological conditions mainly including arthritis [2], spermatogenesis [3], angiogenesis [4] [5], and thrombosis-related disease [6]. Noteworthily, most of these activities are related to proteolytic processing within their C-terminal regions [6]-[9].

ADAMTS-18 has the similar domain organization as other family members. ADAMTS-18 has been shown to be epigenetically silenced in multiple carcinomas and has tumor suppressor activity [10]. Mutation of ADAMTS-18 is strongly associated with colorectal cancer [11]. The data from National Center for Biotechnology Information (NCBI) subject’s gene expression omnibus (GEO) also showed that ADAMTS-18 gene was differentially expressed in subjects with normal skeletal fracture versus subjects with nonunion skeletal fracture [12]. Therefore, it is also associated with bone mineral density (BMD) determination in the major human ethnic groups. Recently, some studies indicate that the ADAMTS-18 gene is also play a crucial role in early eye development [13].

Platelet integrin αIIbβ3 (GPIIb/IIIa) is a heterodimeric receptor of the integrin family expressed at high density (50,000 - 80,000 copies/cell) on the platelet plasma membrane [14]. GPIIIa49-66 (CAPESIEFPVSEAREVLED) is a linear epitope of integrin subunit β3 (GPIIIa) in its extracellular domain. We previously reported that the unique feature of the antibodies (Abs) against GPIIIa49-66 was their ability to induce reactive oxygen species (ROS) through the activation of 12-lipoxygenase and nicotinamide adenine dinucleotide phosphate oxidase (NADPH), leading to complement-independent platelet fragmentation [15] [16]. Recently, we revealed that ADAMTS-18 was the physiologic ligand of platelet GPIIIa49-66 [17]. Thrombin generated from the endothelium of vessel injury is able to cleave ADAMTS-18. The generated ~45-kDa C-terminal cleavage product of ADAMTS-18 becomes activated. It clusters the β3 integrins and induces oxidative platelet fragmentation as we previously described anti-GPIIIa 49 - 66 Ab [17]. We have identified that R775/S776 in spacer region of ADAMTS-18 is one of the potential cleavage sites of thrombin [18]. However, sequence analysis indicates that there still exist two same sites in spacer region neighboring R775/S776 named R823/S824, which also generate similar ~45-kDa C-terminal products in theory when cleaved by thrombin. In this study, we have investigated other thrombin cleavage sites in spacer region of ADAMTS-18 through amino acid mutation and mass spectrometry assay.

2. Materials and Methods

2.1. Reagents

All reagents were obtained from Sigma (St. Louis, MO) unless otherwise designated. Full-length ADAMTS-18 cDNA coding sequence was purchased from ATCC and cloned into mammalian expression vector pBudCE4.1 from Invitrogen (Carlsbad, CA) [17] [18]. ADAMTS-18 peptides were synthesized by Sangon Biotech (Shanghai, China). The in vitro Transcend™ Biotinylated Translation Detection Systems was purchase from Promega (Madison, WI, USA).

2.2. In Vitro DNA Translation and Thrombin Cleavage Assay

Biotinylated-methionine-labeled ADAMTS-18 or its mutant was translated using an in vitro Transcend™ Biotinylated Translation Detection Systems following the protocol provided by the manufacturer. All the peptides or translated proteins were then digested by thrombin or cathepsin D or trypsin according to the protocol provided by the manufacture.

2.3. Immunoblotting

In vitro translation products were separated by 12% SDS/PAGE gels, transferred to a nitrocellulose membrane, and immunoblotted with horseradish peroxide (HRP) conjugated avidin for 1 hour followed by washing with
PBST (0.1% Tween 20). The signal band was detected by chemiluminescence substrate [18].

2.4. Mass Spectrometry

Mass spectrometry was performed as previously described [18]. Briefly, for analysis of ADAMTS-18 cleavage products, a fresh mixture of enzyme and ADAMTS-18 peptide was submitted to molecular weight determination by Matrix assisted laser desorption ionization quadrupole time of flight (MALDI-QTOF) mass spectrometry (MS) (Applied Biosystems 4700 Proteomics Analyzer). To determine the amino acid sequences of newly observed peaks, MS/MS peptide de novo sequencing using a specific software program (Applied Biosystems DeNovo Explorer) was performed.

3. Results

3.1. Thrombin Cleavage of C-Terminal ADAMTS-18 on Several Sites

Previous study has shown that the full-length ADAMTS-18 is proteolyzed by thrombin and results in ~45-kDa C-terminal fragments releasing [17]. The optimal cleavage site for thrombin is R/X [X refers to nonacidic amino acid mainly including R, lys (K), His (H), and Ser (S)] (Figure 1(a)). We have demonstrated that R775/S776 in spacer region of ADAMTS-18 is the potential cleavage site of thrombin [18]. However, analysis of the primary amino acid sequence of ADAMTS18 revealed that there exist three similar thrombin cleavage sites in ADAMTS-18 spacer region named R775/S776, R792/S793 and R823/S824 (Figure 1(b)). The possible molecular weight from these predicted sites to C terminal is ~49-, 47- and 43-kDa, respectively (Figure 1(c)). Therefore, it remained uncertain whether R792/S793 and R823/S824 were also the actual sites of proteolysis.

3.2. Susceptibility of Thrombin for R792/S793 and R823/S824 of C-Terminal ADAMTS-18

To explore these predictions, we synthesized peptides covering the other two putative cleavage sites. The P06594 (PGEFPAGTTFEYQRSFRPERLYAPG) covers R823/S824. The initial molecular weight (MW) of

![Figure 1](image-url)
P06594 is ~3134.5 Da when incubated with PBS buffer (Figure 2(a)). However, thrombin cleaved 27-mer P06594 at R/S site, producing 15-mer N terminal peptide PGEFPAGTTFEYQR (~1746.6 Da) and 12-mer C terminal peptide SFNRPERLYAPG (~1406.6 Da), respectively (Figure 2(b)). The generation of 15-mer peptide becomes obvious when the concentration of thrombin beyond 5 U/ml (Figure 2(c)). Consistently both PBS and thrombin had no effect on P06595 (PGEFPAGTTFEYQRSSSFNRPERLYAPG), in which R823 was mutated to S (Figure 2(d) and Figure 2(e)). The generation of 15-mer peptide completely abrogated when cleaved by various concentrations of thrombin (Figure 2(f)). Hirudin completely inhibited the generation of MW1746.6 and MW1406.6 peak suggesting the specificity of thrombin cleavage (Figure 3). Similar results were obtained with P06728 (ELQVSS SYLAVRSLSQKYLTLGGWSID), which covers R792/S793 producing two peptide peaks (~1717.9 Da and 1351.52) at R792/S793 site (Table 1). We also incubated these peptides with other enzymes cathepsin D or trypsin, and assayed by mass spectrometry. It demonstrated that these cleavage sites are thrombin-specific, and insensitive to cathepsin D or trypsin (Table 1).

3.3. Specificity of Thrombin for Site-Mutated ADAMTS-18 Full-Length Protein

Since R/S775, 792 and 823 in spacer region of ADAMTS-18 are critical for thrombin cleavage, we further constructed mammalian expression vector in which all these susceptible sites were mutated to S/S.775, 792 and 823 (Figure 4(a)).
Figure 2. Susceptibility of thrombin for R823/S824 site of ADAMTS18. (a) (b) Synthesized 27-mer ADAMTS-18 peptide P06594 containing R823/S824 (~3134.5 Da) was incubated with PBS (a) or 5 U/ml thrombin (b) for 1 h. The putative thrombin cleavage site was confirmed by MALDI QTOF mass spectrometry. (c) The releasing of 15-mer N terminal peptide (MW 1746.6) when P06594 was cleaved by different concentration of thrombin at R823/S824 site. (d) (e) Mutated 27-mer ADAMTS-18 peptide P06595 (~3064.5 Da, R823 switches to S823) was incubated with PBS (d) or thrombin (e) in the same condition as P06594 and analyzed by mass spectrometry. Proteolysis after R823 was prevented by substitution of the arginine (Arg, R) to serine (Ser, S). (f) No 15-mer N terminal peptide (MW 1746.6) was released when P06595 was cleaved by different concentrations of thrombin at S823/S824 site.

Bio-methionine-labeled ADAMTS18 and site-mutant ADAMTS-18 were synthesized with in vitro translation system using the expression vector of pBudCe 4.1/ADAMTS-18. Both pBudCe 4.1/ADAMTS-18 (lane 2) and its mutant (lane 4) demonstrated two dominant bands of ~135 kDa and ~75 kDa. The ~135 band represented intact ADAMTS-18 (1221 amino acids), and ~75 kDa band does represent the short form of ADAMTS-18 since luciferase control (lane 1) and the empty vector (lane 3) did not transcribe these two bands (Figure 4(b)). The expression of ~75 kDa short form of ADAMTS-18 is consistent with our previous report [18]. Figure 4(c) demonstrates wide type ADAMTS-18 is proteolyzed by thrombin, and the cleavage fragment is about ~45-kDa. However, thrombin had no effect on mutated ADAMTS-18 in various concentrations (Figure 4(d)).
Table 1. Analysis of thrombin cleavage sites in ADAMTS-18 protein.

<table>
<thead>
<tr>
<th>Number</th>
<th>Peptide location in ADAMTS-18</th>
<th>Sequence</th>
<th>Molecular weight</th>
<th>Molecular weight after thrombin digestion (R/S)</th>
<th>Molecular weight after cathepsin D or trypsin digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>331941</td>
<td>762-791</td>
<td>NEYYPVVIIPAGRSSIEIQELQVSSYLV</td>
<td>3308.41</td>
<td>1447.52 + 1559.3</td>
<td>ND</td>
</tr>
<tr>
<td>331942</td>
<td>762-791</td>
<td>NEYYPVVIIPAGASSIEIQELQVSSYLV</td>
<td>2982.29</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P06728</td>
<td>780-806</td>
<td>ELQVSSYLVARSLSQKYYLTGGWSID</td>
<td>3051.4</td>
<td>1351.52 + 1717.9</td>
<td>ND</td>
</tr>
<tr>
<td>P06729</td>
<td>780-806</td>
<td>ELQVSSYLVSSLSQKYYLTGGWSID</td>
<td>2982.29</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>P06594</td>
<td>808-834</td>
<td>PGEFPAGTTFEYQRSNRPERLYAPG</td>
<td>3314.5</td>
<td>1746.6 + 1406.6</td>
<td>ND</td>
</tr>
<tr>
<td>P06595</td>
<td>808-834</td>
<td>PGEFPAGTTFEYQSSNRPERLYAPG</td>
<td>3066.34</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, no digestion.

Figure 3. Inhibition effect of hirudin on thrombin cleavage. Synthesized 27-mer ADAMTS18 peptide P06594 was incubated with 5 U/ml thrombin and equal amount of hirudin for 1 h and analyzed by mass spectrometry. Representative mass spectrometry map showed proteolysis after R823 was completely inhibited by the addition of hirudin.

Figure 4. Effect of thrombin on ADAMTS-18 mutant. (a) Diagram of ADAMTS-18 mutation sites in which R775, R792 and R823 were simultaneously mutated to S775, S792 and S823. (b) In vitro translation. Lane 1, luciferase (~62 kDa); lane 2, pBudCE4.1/ADAMTS-18 (~135 kDa); lane 3, pBudCE4.1; lane 4, pBudCE4.1/ADAMTS-18 mutant (~135 kDa). (c) Wide-type ADAMTS-18 was incubated with thrombin and analyzed by immunoblotting. Lane 1, Bio-ADAMTS-18 alone; lane 2, Bio-ADAMTS-18 + 5 U/ml thrombin; lane 3, Bio-ADAMTS-18 + 5 U/ml thrombin + hirudin. (d) Bio-ADAMTS18 mutant was incubated with various concentrations of thrombin and analyzed by immunoblotting. Lane 1-4 refers to 5, 10, 20, 30 U/ml thrombin, respectively.
4. Discussion

Despite the similarity shared by ADAMTS family members, most differences among them are found in the C-terminal domains of the protein, suggesting that the C-terminal domains of ADAMTS may determine their in vivo location and substrate specificity [19]-[22]. C-terminal processing has been shown in ADAMTS-1 [19] [21], ADAMTS-4 [20] [22], ADAMTS-8 [4], ADAMTS-9 [5], and ADAMTS-13 [6]. This splicing will shed light on the biological function of these important proteins. Noteworthy, most of cleavage events occur within the spacer region [19]-[22].

We previously reported that cleavage of ADAMTS-18 by thrombin represent a novel mechanism for platelet thrombus clearance [17]. The release of the active 45-kDa C-terminal fragment could regulate thrombus size by inducing oxidative platelet fragmentation. In this study, we first revealed that the R775/S776, R792/S793 and R823/S824 in spacer region of ADAMTS-18 are critical for thrombin cleavage. This cleavage region is similar to those of the ADAMTS family members reported previously [19]-[22]. Physiologically, thrombin is generated rapidly, and at high local concentrations during the normal hemostatic response. We found that ADAMTS-18 was proteolyzed by thrombin at a high thrombin concentration, whereas low thrombin concentration had undetectable cleavage effect on ADAMTS-18 which mimics some physiological conditions, especially platelet thrombus formation. It is of interest in this regard that ADAMTS-13 has recently been shown to be inactivated by thrombin contributing to the loss of ADAMTS-13 VWF cleavage function [6]. Furthermore, ADAMTS-13 has been reported to limit platelet thrombus formation in a shear rate dependent platelet thrombus model on collagen, by its cleavage of ultra large VWF [23].

In present study, we also find ~75-kDa band which is from ADAMTS-18 cDNA in in vitro translation assays. Thrombin or other enzyme (cathepsin D or trypsin) had no effect on the generation of this band. Thus, it is likely other mechanism has been involved in the ADAMTS-18 processing.

5. Conclusion

In summary, this report provides a direct proof that the existence of C-terminal proteolytic cleavage sites of ADAMTS-18 by thrombin, has potential drug application in dissolution of arterial thrombi.

Acknowledgements

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