Regulation of ADAMTS9 Secretion and Enzymatic Activity by Its Propeptide*

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ADAMTS9 is a secreted, cell-surface-binding metalloprotease that cleaves the proteoglycans versican and aggrecan. Unlike most precursor proteins, the ADAMTS9 zymogen (pro-ADAMTS9) is resistant to intracellular processing. Instead, pro-ADAMTS9 is processed by furin at the cell surface. Here, we investigated the role of the ADAMTS9 propeptide in regulating its secretion and proteolytic activity. Removal of the propeptide abrogated secretion of the ADAMTS9 catalytic domain, and secretion was inefficiently restored by expression of the propeptide in trans. Substitution of Ala for Asn residues within each of three consensus N-linked glycosylation sites in the propeptide abrogated ADAMTS9 secretion. Thus, the propeptide is an intramolecular chaperone whose glycosylation is critical for secretion of the mature enzyme. In addition to two previously identified furin-processing sites (Arg74 and Arg287) the ADAMTS9 propeptide was also furin-processed at Arg209. Substitution of Ala for Arg74, Arg209, and Arg287 resulted in secretion of an unprocessed zymogen. Unexpectedly, versican incubated with cells expressing this pro-ADAMTS9 was processed to a greater extent than when incubated with cells expressing wild-type, furin-processable ADAMTS9. Moreover, cells and medium treated with the proprotein convertase inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone had greater versican-cleaving activity than untreated cells. Following furin processing of pro-ADAMTS9, propeptide fragments maintained a non-covalent association with the catalytic domain. Collectively, these observations suggest that, unlike other metalloproteases, furin processing of the ADAMTS9 propeptide reduces its catalytic activity. Thus, the propeptide is a key functional domain of ADAMTS9, mediating an unusual regulatory mechanism that may have evolved to ensure maximal activity of this protease at the cell surface.

ADAMTS9 proteases have critical roles in many biological processes and in inherited and acquired human disorders (1–5). The 19 enzymes of this family share a conserved organization comprising an N-terminal metalloprotease domain and a C-terminal ancillary domain, which contains the thrombospondin type-1 repeats that are the hallmark of the family (6). ADAMTS9 is the largest enzyme of the family, containing 15 thrombospondin type-1 repeats, and its mRNA is widely expressed during embryonic development and in adult tissues (7–9). In previously published work, we showed that when expressed in COS-1 or HEK293F cells, ADAMTS9 is located at the cell surface or within the pericellular matrix (8), suggesting that, despite the lack of a membrane anchor, it could be considered as an operational cell-surface protease. ADAMTS9 can cleave the large aggregating proteoglycans aggrecan and versican (8), suggesting a role in turnover of extracellular matrix. Thus, like its Caenorhabditis elegans ortholog, Gon-1, which is required for cell migration during gonadal morphogenesis (10), it is possible that ADAMTS9 participates in extracellular proteolysis of matrix or cell-surface molecules.

The ADAMTS9 protease domain contains an N-terminal propeptide, which, as in mostzymogens, is believed to maintain enzyme latency and to be proteolytically excised to permit catalytic activity, a process termed “activation.” The ADAMTS9 propeptide contains a number of predicted furin-processing sites, and as in many metalloproteases, propeptide excision is mediated by proprotein convertases (PCs) such as furin. We previously made the unexpected observation that pro-ADAMTS9 was resistant to processing by furin within the secretory pathway but was processed by furin at the cell surface (11). Although a C-terminally truncated zymogen containing only the propeptide and catalytic domain of pro-ADAMTS9 (ProCat) was readily detectable at the cell surface, the furin-processed propeptide and catalytic domain fragments of the zymogen were never found at the cell surface, but instead, were readily detected in the medium. This suggested that the contiguous propeptide and catalytic domain were essential for cell-surface binding and that furin processing either disrupted a cell-binding site or led to a conformational change that resulted in loss of cell-surface binding. Thus, the ADAMTS9 propeptide

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can potentially contribute both to regulation of ADAMTS9 activity and to its localization. In the present work, we continued our investigation into the role of the propeptide in regulating the secretion and activity of ADAMTS9. The results provide further insights on ADAMTS9 propeptide cleavage by protease convertases and demonstrate an unexpected role for the ADAMTS9 propeptide in regulation of enzyme activity.

**EXPERIMENTAL PROCEDURES**

**ADAMTS9 Expression Plasmids and Site-directed Mutagenesis**—Plasmids for the expression of full-length ADAMTS9, or only the signal peptide, propeptide and catalytic domain (Pro-Cat) with C-terminal Myc and His tags were previously described (8, 11). New constructs made for the present studies are shown, as appropriate, in Figs. 1–3. To make an expression plasmid encoding only the propeptide, site-directed mutagenesis was used to convert the P1 Arg of the major furin cleavage site to a stop codon (Arg287 Stop) using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). cDNA encoding the catalytic domain without the prepro-peptide was amplified using Pro-Cat as a template with primers 5'-TCGATTTTTATCTATCCACGGGTGT-TAGAAGTCCTT-3' (with the ClaI site underlined) and 5'-GGAGTCCTACAGGGTTGTTAAGCAAAC-3' (BamHI site underlined and introduced stop codon italicized) and cloned into pFLAG-CMV-1TM (Sigma) for expression with a preprotrypsin leader sequence and N-terminal FLAG tag. A plasmid for expression of ADAMTS9 from the N terminus to the linker 2 peptide (residues 1–1326), named ADAMTS9(N-L2), was generated by PCR and cloned into pcDNA3.1(-)Myc-his A (Invitrogen) for expression with a C-terminal Myc and Hisα tag. Two additional constructs were made for expression of mature ADAMTS9 catalytic domain and mature, nearly full-length ADAMTS9 (ADAMTS9(Cat-L2)) with the endogenous signal peptide, but without the propeptide, by subjecting Pro-Cat and ADAMTS9(N-L2) cDNAs to consecutive rounds of site-directed mutagenesis. In the first round of mutagenesis, an Nhel site was inserted between the propeptide and catalytic domain of each plasmid. In the second mutagenesis step, an Nhel site was inserted between the signal peptide and propeptide of each plasmid. Nhel was used to excise the propeptide lying between these two sites, and the plasmid ends were ligated to obtain constructs that could express the respective polypeptides lacking the propeptide. N-glycosylation sites within the propeptide of Pro-Cat were substituted with Ala (Asn112 (Invitrogen) for expression with a C-terminal Myc and His6 tag.

**Versican Processing Assays**—Conditioned medium was collected from untransfected HEK-293F cells (control) or HEK-293 F cells stably expressing Pro-Cat and dialyzed against phosphate-buffered saline without subsequent concentration. The dialyzed medium was incubated with the thiol-non-cleavable amine-reactive cross-linker bis(sulfosuccinimidyl)suberate (Pierce, final concentration 2 mM according to the manufacturer’s recommendation) for 30 min on ice. The functional groups in this cross-linker are spaced 11.4 Å apart. After blocking unused free functional groups with 50 mM Tris-HCl (pH 7.5) for 5 min, aliquots of the conditioned medium were analyzed by reducing SDS-PAGE and Western blotting with anti-Myc antibody, reducing SDS-PAGE and fluorography, all as previously described (11, 12).

**Cross-linking and Cell Surface Biotinylation**—Conditioned medium was collected from untransfected HEK-293F cells (control) or HEK-293 F cells stably expressing Pro-Cat and dialyzed against phosphate-buffered saline without subsequent concentration. The dialyzed medium was incubated with the thiol-non-cleavable amine-reactive cross-linker bis(sulfosuccinimidyl)suberate (Pierce, final concentration 2 mM according to the manufacturer’s recommendation) for 30 min on ice. The functional groups in this cross-linker are spaced 11.4 Å apart. After blocking unused free functional groups with 50 mM Tris-HCl (pH 7.5) for 5 min, aliquots of the conditioned medium were analyzed by reducing SDS-PAGE and Western blotting with appropriate antibodies. Cell surface biotinylation of cells was done on ice as previously described (11, 13).

**Antibodies, Western Blotting, and Immunoprecipitation**—The RP4 antibody (Abcam, Cambridge, MA) to the ADAMTS9 propeptide was characterized previously and detects an undisclosed peptide epitope (11). Anti-Myc monoclonal antibody 9E10 (Invitrogen) and anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) were obtained from commercial sources. Western blotting was done following reducing or non-reducing SDS-PAGE, using appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham Biosciences-GE Biosciences Healthcare Corp., Piscataway, NJ) for detection. Conditioned medium of stably transfected HEK293F cells was immunoprecipitated with anti-Myc-agarose (Sigma-Aldrich) or with anti-FLAG-agarose (Sigma-Aldrich) as a control followed by reducing SDS-PAGE and immunoblotting with anti-RP4 antibody or anti-Myc antibody. For analysis of N-glycosylation mutants, QBI-293A cells were transiently transfected with wild-type Pro-Cat or the various mutants, metabolically labeled with [35S]Met/Cys mixture for 3 h, followed by immunoprecipitation with anti-Myc, reducing SDS-PAGE and fluorography, all as previously described (11, 12).

**ADAMTS9 Propeptide**
ADAMTS9 Propeptide

ADAMTS9, ADAMTS9 Glu\textsuperscript{335} → Ala, or ADAMTS9 Arg\textsuperscript{74,209,287} → Ala expression vectors, and cultured to confluence in 6-well plates. The cells were changed to 293 SFM-II medium (Invitrogen) and incubated in the presence or absence of 50 μM dec-RVKR-cmk for a further 24 h. The conditioned medium was collected and incubated with versican-rich medium from Marfan skin fibroblasts at a 1:1 ratio at 37 °C for 24 h. The incubated medium was analyzed for versican proteolysis using Western blot analysis as described above. The ADAMTS9 in conditioned medium was evaluated by Western blotting using anti-RP4.

RESULTS

ADAMTS9 Catalytic Domain Lacking the Propeptide Is Not Secreted from Cells—We previously showed that both full-length ADAMTS9 and Pro-Cat (Fig. 1A) were efficiently processed by furin at the surface of HEK293F cells. In the case of Pro-Cat, a 29-kDa unglycosylated catalytic domain (reactive with anti-Myc) and 37-, 22-, and ~20-kDa propeptide fragments (reactive with anti-RP4) were readily detectable in the conditioned medium without concentrating it (Fig. 1B). We previously showed that little or no intactzymogen was secreted into conditioned medium of HEK293F cells (e.g. Fig. 1B), and moreover, no processed fragments were present at the surface of these cells, indicating their immediate release upon furin processing (11). Because furin-processed full-length ADAMTS9 is difficult to detect in conditioned medium because of its large size and C-terminal processing, which removes the Myc tag, we studied the biosynthetic profile of a shorter form of the precursor, ADAMTS9(N-L2) (Fig. 1A). This precursor was detected in transfected HEK293F cells as a single 125-kDa species reactive with both anti-Myc and anti-RP4 (Fig. 1C), whereas the furin-processed Cat-L2 (~100 kDa) and the 37-, 22-, and 20-kDa propeptide fragments were seen in conditioned medium using anti-Myc and anti-RP4, respectively (Fig. 1C). To examine the role of the propeptide in regulating secretion, a construct for expression of the catalytic domain alone with a proprotease signal peptide and N-terminal FLAG tag, was generated (Fig. 2A). The FLAG-tagged catalytic domain was not secreted into the conditioned medium of HEK293F cells, although it was present in the cell lysate (Fig. 2A, left-hand panel). However, the propeptide alone (ADAMTS\textsubscript{9-N-L2}) was efficiently secreted into the conditioned medium, and underwent furin processing to generate fragments similar to those previously seen upon Pro-Cat expression (Fig. 2A, right-hand panel).

Co-transfection of the propeptide with the FLAG-tagged catalytic domain failed to rescue its secretion (Fig. 2A, left-hand panel). These experiments suggested that the propeptide was essential for secretion of ADAMTS9 but that it was unable to rescue secretion of the mature enzyme when provided in trans. To exclude potential interference of the N-terminal FLAG tag in secretion, as well as to investigate whether a longer form of ADAMTS9 could be secreted without the propeptide, we repeated these experiments using additional constructs in which in addition to lacking the N-terminal FLAG-tag, utilized the endogenous signal peptide of ADAMTS9 (Fig. 2B). Both ADAMTS9(Cat) and ADAMTS9(Cat-L2) were detectable within the cell lysate (Fig. 2B). Co-expression of the isolated propeptide diminished the levels of each construct within the cell lysate (Fig. 2B) but could not restore their secretion to the robust levels seen in conditioned medium upon expression of the Pro-Cat or ADAMTS9(N-L2) constructs (Fig. 1, B and C). For example, the catalytic domain derived from Pro-Cat is robustly detected upon Western blot analysis of 20 μl of uncentrated conditioned medium from transfected cells, requiring only a few seconds of exposure to ECL reagent (e.g. Fig. 1B). However, the Myc-tagged isolated catalytic domain co-expressed with propeptide was only detectable following acetone precipitation of 500 μl of medium and long exposure (5 min) of the immunoblot to ECL reagent (Fig. 2B). The Cat-L2 construct was undetectable in the medium following co-expression with the propeptide (Fig. 2B). Collectively, these experiments establish: (i) that the propeptide is essential for secretion of ADAMTS9 and (ii) that the propeptide inefficiently restores
secretion of the mature enzyme when provided in trans, demonstrating that it can function as a weak chaperone in trans, but that, for such function to be optimal, it is required in cis.

N-Linked Oligosaccharide in the Propeptide Is Essential for Secretion—Treatment of ADAMTS9 Pro-Cat with peptide N-glycosidase F previously demonstrated that the propeptide, but not the catalytic domain, was N-glycosylated, as predicted by the primary sequence (11). Because N-linked oligosaccharides are predicted to be attached to Asn112, Asn135, and Asn271 (these residues are located within the consensus motif Asn-Xaa-Ser/Thr, where Xaa is any residue except proline (14)) and may be involved in regulating correct folding of secreted proteins (15), we mutated these residues individually and in combination by substituting Asn with Ala in Pro-Cat. Pro-Cat is present in transfected QBI-293A cells as a major glycosylated molecular species of 60 kDa and a 50-kDa unglycosylated species (8) (Fig. 3, upper panel). Protein expressed from each of the single Asn mutant Pro-Cat plasmids was detected in the respective transfected cell lysate but migrated more rapidly than the fully glycosylated Pro-Cat, suggestive of a lack of glycosylation at that site (Fig. 3, upper panel). Pro-Cat in which all three putative sites were mutated showed a major band, which migrated at the level of the unglycosylated Pro-Cat (Fig. 3, upper panel). Together, these observations demonstrated that the secreted ADAMTS9 propeptide is constitutively glycosylated at Asn112, Asn135, and Asn271 as suggested by previous analysis (11). Although the 29-kDa furin-processed catalytic domain fragment was present in conditioned medium of wild-type Pro-Cat-transfected cells, the catalytic domain derived from N-glycosylation-defective proteins was not found in the media of transfected cells (Fig. 3, lower panel). This suggests that N-linked glycosylation at each site within the propeptide is essential for secretion of ADAMTS9 zymogen and as a corollary, that secreted ADAMTS9 normally contains an oligosaccharide at each of these sites.

Propeptide Remains Non-covalently Associated with the Catalytic Domain after Furin Processing—Previous studies using reducing SDS-PAGE of conditioned medium from ADAMTS9-transfected cells demonstrated that the propeptide was secreted into conditioned medium following proteolysis of the zymogen at the cell surface (11). To investigate whether the propeptide interacted with the catalytic domain after processing or was secreted as a discrete entity, the conditioned medium derived from N-glycosylation-defective proteins was not found in the media of transfected cells (Fig. 3, lower panel). This suggests that N-linked glycosylation at each site within the propeptide is essential for secretion of ADAMTS9 zymogen and as a corollary, that secreted ADAMTS9 normally contains an oligosaccharide at each of these sites.

![FIGURE 2. The ADAMTS9 propeptide is essential for secretion of the catalytic domain.](image-url)

**A**, secretable FLAG-tagged catalytic domain or the ADAMTS9 propeptide were expressed individually or together in HEK293F cells using plasmids expressing the constructs shown at the top. Immunoblotting of cell lysate (Cell) and conditioned medium (CM) was done using anti-FLAG (left-hand panel) or anti-RP4 (right-hand panel). Note that the propeptide (P) expressed in isolation is secreted into the CM (right-hand panel), but the catalytic domain (C) is not (left-hand panel). Secretion of the catalytic domain is not rescued by co-expression of the propeptide. Molecular mass markers are shown to the right of each panel. **B**, expression of C-terminal Myc-tagged Cat (Cat-Myc) or ADAMTS9(Cat-L2) and their co-expression with the propeptide in HEK293F cells. Immunoblotting of cell lysate and conditioned medium (CM) was done using anti-Myc. ADAMTS9(Cat-L2) and catalytic domain (C) are indicated. The Cat-L2 construct was used in preference to full-length ADAMTS9, because it is more resistant to C-terminal proteolysis and thus more readily detected in the medium of transfected cells. Note a weakly reactive catalytic domain band in acetone-precipitated conditioned medium following co-expression of propeptide but no secretion of ADAMTS9 (Cat-L2). The 35- and 48-kDa bands are seen in all lanes and are nonspecific.
### ADAMTS9 Propeptide

**FIGURE 3. N-Linked glycosylation is essential for Pro-Cat secretion.** Q8I293A cells were transiently transfected with Pro-Cat or the Asn (N) mutants introduced into the N-linked glycosylation sites shown at the top of the figure, followed by incubation with [³⁵S]Met + Cys amino acids for 3 h, and immunoprecipitation of labeled proteins with anti-Myc, SDS-PAGE, and fluorography. Untransfected cells were used as a control. Note the reduction in size of each mutant protein in the cell lysate and the absence of the corresponding catalytic domain in the conditioned medium (CM). The glycosylated and unglycosylated zymogen and catalytic domain (C) are indicated. Molecular mass markers are shown on the right.

| N^112LT | N^135ST | N^2771KT |
|---------|---------|---------|
| Pro     | Myc     | Cat     |

**FIGURE 4. Association of the cleaved propeptide with the catalytic domain.** A, conditioned medium of HEK293F cells stably transfected with Pro-Cat_{myc-His} was immunoprecipitated with anti-Myc-agarose or anti-FLAG-agarose (as a negative control) followed by immunoblotting (IB) with the indicated antibodies. Note that anti-Myc immunoprecipitates propeptide fragments (left-hand panel). B, non-reducing SDS-PAGE followed by immunoblotting with anti-RP4 or anti-Myc shows that the propeptide (P) and catalytic domain (C) do not associate under non-reducing conditions, suggesting a non-covalent, possibly ionic, interaction. A small amount of complexed propeptide and catalytic domain (P-C) is indicated. C, cross-linking of cleaved propeptide and catalytic domain in conditioned medium of Pro-Cat-transfected cells. Conditioned medium was treated with the non-reducible cross-linker bis(sulfo)iminodisuccinimidyl) suberate (BS3). Analysis of conditioned medium by reducing SDS-PAGE and immunoblotting with anti-RP4 and anti-Myc shows that the cleaved propeptide (P) and catalytic domain (C) migrate as cross-linked species (P-C) whose major forms are larger than the zymogen (Z), for reasons that are presently unclear. D, cell-free conditioned medium (CM) from HEK293F cells expressing Pro-Cat was incubated at 37°C for the indicated durations. Western blot analysis was done using anti-RP4.

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The propeptide, but also the smaller 20- to 22-kDa fragments derived from it (Fig. 4A). As a control for these experiments, immunoprecipitation using anti-FLAG agarose, which does not recognize this construct, did not provide any anti-RP4 immunoreactivity (Fig. 4A). We noted that the propeptide was not co-immunoprecipitated with the catalytic domain under conditions where detergents were used for washing the protein-A beads (e.g. Fig. 3). In the immunoprecipitation experiments shown in Fig. 4A, the beads were washed several times with phosphate-buffered saline, and no detergents were present. Furthermore, non-reducing SDS-PAGE analysis of the medium showed discrete propeptide and catalytic domain fragments as the major immunoreactive species, although low levels of a propeptide-catalytic domain complex were also detectable (Fig. 4B). This demonstrates that the complex between the propeptide and catalytic domain in the medium is not disulfide-bonded. Taken together, this suggested that the binding of the propeptide to the catalytic domain is not only non-covalent but is also susceptible to dissociation in the presence of detergents such as SDS. Next, cross-linking of the secreted propeptide and catalytic domain was done at low concentrations of the target proteins (unconcentrated medium) using a cross-linker with a short arm (11.4 Å) between the functional groups. Fig. 4C shows that the propeptide and catalytic domain were efficiently cross-linked with very little residual isolated catalytic domain or propeptide. The molecular mass of the major complexes (100–250 kDa) reactive with both anti-Myc and anti-RP4 is considerably larger than the zymogen (60 kDa) (Fig. 4C). It is possible that other molecules may be included within the complex, but resolution of this issue is beyond the scope of the present work. Cell-free incubation of conditioned medium containing the propeptide and catalytic domain revealed that the propeptide fragments were quite stable over a period of 24 h (Fig. 4D). The amount of the 37-kDa fragment decreased slowly as a function of time, possibly by conversion to the smaller propeptide fragments, as we previously noted (11). Taken together, these data demonstrate that the propeptide and catalytic domain are in close proximity to each other following furin processing and that the resulting complex is quite stable.
The substitution of Ala for Arg209 led to the disappearance of the 20-kDa proteolytic fragment of the propeptide in conditioned medium (data not shown), although it did not affect processing at the downstream processing site, Arg287 (Fig. 5B). Moreover, substitution of Arg209,287 with Ala abolished zymogen processing (Fig. 5B), although low levels of unidentified proteolytic fragments were occasionally seen (e.g. Fig. 6D). Thus, the substitution of Arg74, Arg209, and Arg287 could be used to produce an essentially uncleavable zymogen.

Furin-processed ADAMTS9 Zymogen Cleaves Versican Inefficiently—To ask whether the ADAMTS9 propeptide had a role in regulating its latency and enzymatic activity, or if the bound propeptide fragments following furin-processing affected catalytic activity, we compared versican processing by full-length ADAMTS9, full-length ADAMTS9 Arg74,209,287 → Ala and full-length ADAMTS9 Glu435 → Ala. The Glu435 → Ala mutant replaces the critical catalytic Glu residue in the active site and is predicted to generate a proteolytically inactive enzyme (19, 20). Because purification of full-length ADAMTS9 has not been successful despite numerous attempts, and because purified Pro-Cat could not cleave versican (8), these experiments were first done using a cell-based assay. In this assay, HEK293F cells are transiently transfected with the various constructs described above and incubated with medium obtained from fibroblasts expressing high levels of versican. Untransfected cells were used to ascertain baseline, endogenous levels of versican proteolysis. Because ADAMTS9 binds to the cell surface (8), a cell-based assay also provides the appropriate context for testing proteolysis, including putative cofactors (21). The V1 isoform of versican, the predominant form made by fibroblasts, is proteolytically processed by some members of the ADAMTS family, including ADAMTS9, within the sequence DPEAAE441A442RRGQ to generate a 70-kDa fragment reactive with the anti-DPEAAE antibody (8, 22). This antibody specifically detects the neo-epitope generated by cleavage at 441EA442 site, but it does not react with uncleaved versican core protein (8, 22). Untransfected HEK293F cells have low background levels of versicanase activity (Fig. 6A, upper panel). Unexpectedly, cells expressing the ADAMTS9 Arg74,209,287 → Ala mutant showed more robust processing of versican at the 441EA442 site than the wild-type ADAMTS9 (Fig. 6A, upper panel). ADAMTS9 Glu435 → Ala could not generate an immunoreactive fragment, because this mutation renders the protease domain catalytically inactive (Fig. 6A, upper panel). The three constructs were expressed at comparable levels in cells (Fig. 6A, middle panel) and were equally represented at the cell surface as shown by cell-surface biotinylation (Fig. 6A, lower panel). This observation suggests that furin processing of the propeptide could decrease the versicanase activity present in pro-ADAMTS9. Because of concerns about the possible structural consequences of mutating three Arg residues, we undertook parallel experiments in which HEK293F cells were treated with 50 μM dec-RVKR-cmk, which effectively prevented processing of pro-ADAMTS9 (Fig. 6B). Cells expressing ADAMTS9 in the presence of 50 μM dec-RVKR-cmk also showed greater activity toward versican than cells expressing the processable ADAMTS9 (Fig. 6C, upper panel). dec-RVKR treatment affected neither the cellular levels of ADAMTS9 zymogen nor its localization at the cell surface (Fig. 6C). In another variation of the cell-based assay, we used conditioned medium from cells

![Image](49x198 to 408x733)

**FIGURE 5. Identification of a novel PC-processing site in the ADAMTS9 propeptide and generation of PC-uncleavable ADAMTS9 Pro-Cat.** A, the domain organization of ADAMTS9 Pro-Cat is shown with expansion of sequence motifs containing two previously identified processing sites and a putative novel PC-processing site (RR209S) in the propeptide. B, Western blotting (using anti-Myc) of conditioned medium from HEK293F cells transfected with wild-type Pro-Cat or various mutants as indicated. Untransfected cells were used as a control. The zymogen (Z) and catalytic domain (Cat) are indicated. The residues comprising the various fragments are identified on the right. The 32-kDa fragment is presently unidentified. Note that expression of mutant Pro-Cat374/209/287A results in unprocessed zymogen.
ADAMTS9 Propeptide

![Figure 6](image-url)

**FIGURE 6.** Furin processing of the ADAMTS9 propeptide diminishes versicanase activity. A, HEK293F cells expressing full-length ADAMTS9, uncleavable zymogen (ADAMTS9142/209/287A), or ADAMTS9 with a Glu435→Ala mutation in the active site (ADAMTS9E435A) were incubated with medium enriched in versican. Neo-epitope antibody (anti-DPEAAE) was used to identify proteolytic cleavage on immunoblots as indicated (top panel). Immunoblotting with anti-RP4 demonstrates comparable levels of ADAMTS9 expressed in cells (middle panel), and cell-surface biotinylation shows equivalent levels at the cell surface (lower panel). The result is representative of four independent experiments. B, inhibition of zymogen processing by 50 μM dec-RVKR-cmk. Note that only the zymogen (Z) is seen in the conditioned medium of the treated cells upon Western blotting using anti-RP4, and propeptide fragments (P) are only seen without treatment. C, versican processing by ADAMTS9-transfected HEK293F cells treated with dec-RVKR-cmk is greater than untreated cells. Neo-epitope antibody (anti-DPEAAE) was used to identify proteolytic cleavage on immunoblots as indicated (top panel). Immunoblotting with anti-RP4 demonstrates comparable levels of ADAMTS9 expressed in cells (middle panel), and cell-surface biotinylation suggests equivalent levels at the cell surface. The result is representative of four independent experiments. D, conditioned medium was collected for versican proteolysis assays from untransfected cells, cells transfected with the constructs shown, or cells expressing wild-type ADAMTS9 treated with dec-RVKR-cmk. The upper panel is an immunoblot using the versicanase neo-epitope antibody. The lower panel is an immunoblot with anti-RP4 to show the comparative levels and processing status of ADAMTS9. The identity of the small RP4-reactive bands in ADAMTS9142/209/287A-transfected cells is unknown; however, note that the majority of immunoreactive ADAMTS9 in this lane is in zymogen form.

expressing the various constructs or following treatment with dec-RVKR-cmk. The results (Fig. 6D), further confirm that intact ADAMTS9 zymogen, but not furin-processed ADAMTS9 has proteolytic activity against versican. Although the data strongly suggest that furin processing of pro-ADAMTS9 reduces its versicanase activity, we cannot presently exclude the formal possibility that this effect of ADAMTS9 in the cell-based assays is an indirect one that results from its effects on another protease.

**DISCUSSION**

Propeptides of protease zymogens have been shown to have important, if transient, functions in inhibition of catalytic activity (23). Proteolytic processing of propeptides has been studied in detail in the MMP and ADAM families as well as in some ADAMTS proteases. In most secreted, soluble MMPs (e.g. pro-MMP3 and pro-MMP7), the propeptide binds to the active site zinc atom through a free sulphydryl group and maintains the enzyme in a latent, inactive state (24). This bond is destabilized by propeptide proteolysis (24) or chemical modification, which occurs extracellularly, and is not proprotein convertase-mediated (25). In these MMPs, removal of the propeptide is an important prerequisite for obtaining enzymatic activity (24). In the case of MMP14 (membrane-type 1 MMP), an MMP that is processed and activated by furin (26), the propeptide is excised in the trans-Golgi, and constitutively active MMP14 is transported to the cell surface. The MMP14 propeptide is essential as an intramolecular chaperone, because MMP14 lacking the propeptide is not secreted. However, unlike ADAMTS9, expression of the propeptide in trans restores secretion (27). The propeptides of a number of ADAMs (e.g. ADAM9, ADAM12, and ADAM17) (28–30) are constitutively excised in the secretory pathway by furin. The ADAM17 propeptide, like MMP14 and ADAMTS9, is essential for secretion, and like MMP14, it can function as a chaperone in trans to enable secretion (30, 31). Recently, analysis of ADAM12 showed that its propeptide remained associated with the catalytic domain after processing of the zymogen by furin (32), but whether this is a general characteristic of the ADAM family is not known.
In particular, the cell-surface processing of pro-ADAMTS9 led us to ask what role the propeptide had in secretion andzymogen activation.

The data provided here make several novel observations, some of which may be potentially of broad relevance to the ADAMTS proteases. First, the present studies demonstrate that, unlike ADAMTS13 (33), the propeptide is an absolute requirement for ADAMTS9 secretion. Second, the critical role of glycosylation demonstrated here suggests that correct modification of the propeptide is essential for its function as an intramolecular chaperone. Third, in attempting to generate a zymogen that was resistant to furin processing, we characterized a previously unknown, atypical furin-processing site. Mutation of this site and of two previously identified sites was successful in generating a furin-resistant zymogen. Finally, and most significantly, we showed that furin processing of ADAMTS9 reduces its proteolytic activity toward versican.

Furin processing of metalloproteasezymogens is generally referred to as “activation,” because it typically transforms a latent enzyme activity into a functional proteolytic activity. Consistent with this, the ADAMTS4 propeptide was found to have an inhibitory role in the zymogen, because the ADAMTS4 zymogen lacked proteolytic activity against aggrecan (38). However, retention of the propeptide did not affect the ability of ADAMTS13 to process von Willebrand factor, and moreover, this propeptide was dispensable for folding (33). The ADAMTS13 propeptide is not just unusually small, but unlike the other ADAMTS propeptides, it contains only two cysteine residues (versus three for the others) (33); thus, it is not an appropriate model for the other 18 ADAMTS proteases.

Our observation that furin processing of the ADAMTS9 propeptide resulted in loss of proteolytic activity against versican was unexpected and, to our knowledge, is without precedent in the protease field. We are aware of two other examples in the literature in which furin processing was associated with loss of enzymatic activity (40, 41), but in both these instances cleavage occurred at sites unrelated to the propeptide. The mechanism by which furin processing reduces ADAMTS9 catalytic activity is presently unclear. A potential mechanism is suggested by our observation that the propeptide and its fragments remain attached to the catalytic domain following processing. The immediate release of furin-processed Pro-Cat from the cell surface (11) suggests that furin cleavage induces a conformational change in Pro-Cat. We speculate that such a conformational shift may cause the propeptide or its fragments to block the active site and thus hinder proteolysis of versican (Fig. 7). Note, however, that instantaneous release into the conditioned medium does not occur when full-length ADAMTS9 is furin-processed, because the ancillary domain provides additional anchorage to the cell surface. Close contact between the propeptide and catalytic domain is suggested both by effective cross-linking using a short-arm cross-linker in unconcentrated medium, and by the efficient disappearance of individual propeptide and catalytic domain fragments from reducing SDS-PAGE following cross-linking. The contact between propeptide and catalytic domain likely occurs at several sites, because the derivative fragments of the propeptide are immunoprecipitated with the catalytic domain and cross-linked to it.

A hypothetical mechanism for the loss of proteolytic activity upon furin processing is schematically depicted in Fig. 7. The following experimental evidence supports this model: First, that furin-processed Pro-Cat shows immediate loss of cell-surface binding, suggestive of a conformational change, second, that all propeptide fragments are closely associated with the catalytic domain after furin processing, and third, that the furin-processed pro-ADAMTS9 has less versicanase activity. Clarification of the structural basis for this model is beyond the scope of the present body of work, and it is also not clear what role the potentially unpaired cysteine in the propeptide plays in this regulatory mechanism. The stable association of the propeptide with the mature ADAMTS9 molecule is of additional potential significance, because it may indicate a possible role for the propeptide in mediating subsequent intermolecular interactions as a distinct domain of ADAMTS9. The stability of the secreted propeptide in association with mature ADAMTS9 also suggests the possibility that it could be useful as a marker for this protease. Thus, taken together, with previous work (11), it is clear that the ADAMTS9 propeptide constitutes a critical functional domain of this protease, because it has essential roles in secretion, cell-surface binding, as well as maintenance of enzyme activity.

Propositional Functional Significance of the Unusual Mechanism and Consequence of Pro-ADAMTS9 Processing by PCs—Why is ADAMTS9 handled in such an unusual fashion by cells? We propose that these unusual features constitute a mechanism that ensures that ADAMTS9 activity is maximal at the cell surface. Thus, by a yet-unknown mechanism, pro-ADAMTS9 is protected from furin processing until it reaches the cell surface, ensuring that proteolytic activity is not lost prematurely. Unprocessed pro-ADAMTS9 is retained at the cell surface (11), but once it is processed there, its activity is reduced or lost. Thus maximum catalytic activity, at least against versican, is present at the cell surface. This phenomenon, taken together with the role that the ADAMTS9 homologs Gon-1 and ADAMTS20 have in cell migration (10, 42) and its clearly demonstrated localization at the cell surface (8, 11), strongly suggests that ADAMTS9 is operationally a cell-surface protease. The life cycle of a proteolytic activity subject to furin-dependent inactivation, such as of ADAMTS9, is predicted to be short. Furthermore, function of a protease that is regulated in this unusual fashion is likely to be highly dependent on transcriptional regulation of its mRNAs. Indeed, developmental analysis has shown ADAMTS9 to be very dynamically regulated at the transcriptional level and to be strongly associated with cell populations actively undergoing migration, such as trophoblast giant cells, endothelial cells, and neural crest cells (7).
ADAMTS9 Propeptide

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