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Abstract: Proteolysis of the Glu(441)-Ala(442) bond in the glycosaminoglycan (GAG) domain of the versican-V1 variant by a disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif (ADAMTS) proteases is required for proper embryo morphogenesis. However, the processing mechanism and the possibility of additional ADAMTS-cleaved processing sites are unknown. We demonstrate here that if Glu(441) is mutated, ADAMTS5 cleaves inefficiently at a proximate upstream site but normally does not cleave elsewhere within the GAG domain. Chondroitin sulfate (CS) modification of versican is a prerequisite for cleavage at the Glu(441)-Ala(442) site, as demonstrated by reduced processing of CS-deficient or chondroitinase ABC-treated versican-V1. Site-directed mutagenesis identified the N-terminal CS attachment sites Ser(507) and Ser(525) as essential for processing of the Glu(441)-Ala(442) bond by ADAMTS5. A construct including only these two GAG chains, but not downstream GAG attachment sites, was cleaved efficiently. Therefore, CS chain attachment to Ser(507) and Ser(525) is necessary and sufficient for versican proteolysis by ADAMTS5. Mutagenesis of Glu(441) and an antibody to a peptide spanning Thr(432)-Gly(445) (i.e. containing the scissile bond) reduced versican-V1 processing. ADAMTS5 lacking the C-terminal ancillary domain did not cleave versican, and an ADAMTS5 ancillary domain construct bound versican-V1 via the CS chains. We conclude that docking of ADAMTS5 with two N-terminal GAG chains of versican-V1 via its ancillary domain is required for versican processing at Glu(441)-Ala(442). V1 proteolysis by ADAMTS1 demonstrated a similar requirement for the N-terminal GAG chains and Glu(441). Therefore, versican cleavage can be inhibited substantially by mutation of Glu(441), Ser(507), and Ser(525) or by an antibody to the region of the scissile bond.

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Determinants of Versican-V1 Proteoglycan Processing by the Metalloproteinase ADAMTS5*

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Background: The mechanisms of versican proteolysis by ADAMTS proteases are unknown.

Results: The ADAMTS5 ancillary domain and specific chondroitin sulfate chains of versican are required for proteolysis.

Conclusion: Docking between the ADAMTS5 ancillary domain and CS chains is a major mechanism underlying versican proteolysis. Proteolysis by ADAMTS1 has a similar requirement for GAG chains.

Significance: The findings suggest strategies for blocking versican cleavage.

Proteolysis of the Glu441-Ala442 bond in the glycosaminoglycan (GAG) β domain of the versican-V1 variant by a disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif (ADAMTS) proteases is required for proper embryo morphogenesis. However, the processing mechanism and the possibility of additional ADAMTS-cleaved processing sites are unknown. We demonstrate here that if Glu441 is mutated, ADAMTS5 cleaves inefficiently at a proximate upstream site but normally does not cleave elsewhere within the GAGβ domain. Chondroitin sulfate (CS) modification of versican is a prerequisite for cleavage at the Glu441-Ala442 site, as demonstrated by reduced processing of CS-deficient or chondroitinase ABC-treated versican-V1. Site-directed mutagenesis identified the N-terminal CS attachment sites Ser507 and Ser525 as essential for processing of the Glu441-Ala442 bond by ADAMTS5. A construct including only these two GAG chains, but not downstream GAG attachment sites, was cleaved efficiently. Therefore, CS chain attachment to Ser507 and Ser525 is necessary and sufficient for versican proteolysis by ADAMTS5. Mutagenesis of Glu441 and an antibody to a peptide spanning Thr432-Gly445 (i.e. containing the scissile bond) reduced versican-V1 processing.

ADAMTS5 lacking the C-terminal ancillary domain did not cleave versican, and an ADAMTS5 ancillary domain construct bound versican-V1 via the CS chains. We conclude that docking of ADAMTS5 with two N-terminal GAG chains of versican-V1 via its ancillary domain is required for versican processing at Glu441-Ala442. V1 proteolysis by ADAMTS1 demonstrated a similar requirement for the N-terminal GAG chains and Glu441. Therefore, versican cleavage can be inhibited substantially by mutation of Glu441, Ser507, and Ser525 or by an antibody to the region of the scissile bond.

Versican is a member of a family of large aggregating proteoglycans (termed lecticans) present in the extracellular matrix that includes aggrecan, brevican, and neurocan (1). These proteoglycans have a similar structure, comprising a globular N-terminal domain (G1), a central glycosaminoglycan (GAG)2 domain containing attachment sites for GAG chains, and a globular C-terminal domain (G3) (2). Aggrecan alone has an additional globular domain (G2) that lies between the G1 and G3 domains on the N-terminal side of its GAG domain. The predominant GAG present in these proteoglycans is chondroitin sulfate (CS). Through their G1 domains, these proteoglycans bind to hyaluronan, an interaction that is stabilized by link protein, forming large multimeric complexes that interact with cell surface hyaluronan receptors such as CD44 (3–5). The versican G3 domain binds to fibulin 1 and 2, fibrillin 1, and tenasin C and R (6–8). Versican is a component of the pericellular matrix as well as the interstitial extracellular matrix networks (9–11). Versican is widely expressed in embryonic and extraembryonic tissues (12–14), whereas aggrecan is specific for cartilage and the central nervous system, and brevican and neurocan are only found in the central nervous system (1). As a component of embryonic cardiac jelly, the interdigit mesenchyme, and the prechondrogenic mesenchyme of the limb, hyaluronan-versican complexes have crucial roles in embryogenesis (3, 15–22) and in regulating cell migration, proliferation, and apoptosis (9, 20, 23–25). Versican is present in adult vasculature, skin, and the central nervous system, as well as in solid tumors of several organs (26–29). Versican has several splice isoforms arising from alternative splicing of two large exons encoding CS attachment domains, termed GAGα and GAGβ (30–32). These variants are V0 (containing GAGα and GAGβ), V1 (containing GAGβ only), V2 (containing GAGα only), and V3, lacking either GAG domain. V2 is primarily found in the central nervous system and V0 in the nervous and...
Versican Processing by ADAMTS5

cardiovascular systems, but V1 is widely expressed, especially in the embryo (12, 13, 26, 33–36). V3 expression and localization are poorly defined. Recently, a new variant, V4, generated by splicing at a cryptic site within the GAGβ domain, has been identified in cancer cells (28).

ADAMTS proteases cleave aggrecan at multiple sites, an activity named aggrecanase, which is a major contributor to cartilage destruction in osteoarthritis (37–39). Among these sites, one within the aggrecan G1-G2 interglobular domain (Glu$^{374}$-Ala$^{375}$) was deemed critical because it released the entire GAG domain (40). Additional ADAMTS cleavage sites have been identified within the GAG-bearing domain (39). Although aggrecanase sites lack a sequence consensus, Sandy et al. (35) noticed a preference for ADAMTS cleavage after glutamate residues and predicted a cleavage site in versican-V1 corresponding to the aggrecan interglobular domain by comparison of versican and aggrecan core protein sequences. They generated a neoepitope antibody recognizing the predicted new C terminus generated after ADAMTS cleavage, i.e. the sequence DPEAAE$^{441}$ (corresponding to DPEAAE$^{1428}$ in V0) (35).

The predicted scissile bond Glu$^{441}$-Ala$^{442}$ was cleaved by ADAMTS1 and ADAMTS4, and this versican activity has been detected in the aortic intima (35). Subsequently, ADAMTS5, ADAMTS9, ADAMTS15, and ADAMTS20 have been found to cleave this site (41–43). Analysis of mice lacking Adamts1, Adamts5, Adamts9, and Adamts20 identified anomalies in ovulation, interdigital web regression, skin pigmentation, cardiac development, and palate formation that were associated with reduced versican processing (16, 17, 19, 33–48). The N-terminal V1 fragment extending to DPEAAE$^{441}$ and now termed versikine (19) induced apoptosis in ADAMTS5+ Adamts20-deficient interdigital webs, which failed to undergo regression because of reduced extracellular matrix breakdown and apoptosis (17). Therefore, a major physiological role that has emerged for several ADAMTS proteases is processing of versican during embryogenesis, although it remains unclear whether versican is the only substrate that explains developmental defects in ADAMTS gene mutants.

Despite the exceptional biological relevance of versican processing by ADAMTS proteases, it is a poorly understood process. Among the questions that have not been addressed are whether versican is cleaved at additional sites in the core protein and which molecular determinants in versican or ADAMTS proteases are crucial for the enzyme-substrate interaction and proteolysis. This knowledge would offer potential means to block versican processing as a way of further investigating the biological relevance of versican processing. Collectively, these unresolved questions motivated this analysis of versican-V1 processing by ADAMTS5.

EXPERIMENTAL PROCEDURES

ADAMTS and Versican Expression Plasmids and Site-directed Mutagenesis—Mammalian expression plasmids for ADAMTS1 and ADAMTS5 expression have been described previously (41, 49). A versican-V1 plasmid in vector pSecTagA (Invitrogen), the versican V4 expression plasmid, and the G1-DPEAAE plasmid made by inserting a stop codon after Glu$^{441}$ have been published previously (17, 28, 50). The V1 expression plasmid had an intervening 3′-untranslated sequence between the stop codon and the epitope tags. Therefore, an Xhol restriction site was inserted to disrupt the stop codon using the QuikChange mutagenesis kit (Stratagene, Santa Clara, CA), the 3′-untranslated sequence was excised, and the plasmid was religated to render the versican ORF continuous with the myc and His$_{6}$ tags. To generate the constructs V-5GAG-myc, V-2GAG-myc, and DPEAAE-myc (Fig. 1A), a second Xhol site was placed at the appropriate location within the versican ORF. Mutagenized plasmids were digested with Xhol, and the region between the two Xhol sites was eliminated by agarose electrophoresis followed by religation of the plasmids. Specific glycerol or serine residues within four N-terminal GAG attachment sites (i.e. Ser-Gly or Gly-Ser motifs within an acidic sequence consensus) (2) in the V-5GAG construct were mutated by site-directed mutagenesis (Ser$^{507}$ to Ala, Ser$^{525}$ to Gly, Gly$^{645}$ to Val, and Ser$^{655}$ to Ala). Residues around the Glu$^{441}$-Ala$^{442}$ scissile bond were mutated using the QuikChange mutagenesis kit (Stratagene). All introduced mutations were verified by nucleotide sequencing.

Cell Culture, Transfections, and Enzymatic Deglycosylation—HEK293F cells (ATCC) were cultured in DMEM supplemented with 10% FBS and antibiotics. CHO-K1 and pgsA-745 cells (ATCC) (51) were cultured in 1:1 Ham’s F12 and DMEM supplemented with 10% FBS and antibiotics. ADAMTS and versican expression plasmids were transiently transfected or cotransfected using FuGENE6 (Roche Diagnostics). Conditioned medium from empty vector (pcDNA3.1 MycHis, Invitrogen)-transfected cells was used as the control in versican digests. Serum-free medium was collected from transfected cells after 48 h. Cells were lysed in 1% (w/v) Triton X-100, 10 mM Tris HCl (pH 7.6) containing complete protease inhibitor mixture (Roche Diagnostics) to obtain a cell lysate. To detect N-glycosylation of the versikine-myc construct, it was reduced by addition of 2% 2-mercaptopetoanol and boiling for 5 min prior to incubation with peptide N-glycanase F (New England Biolabs, Ipswich, MA) for 2 h at 37 °C. Unless specified otherwise, reagents were from Sigma-Aldrich (St. Louis, MO).

Generation of Anti-VC, a Cleavage-blocking Versican Polyclonal Antibody—Anti-VC was generated in rabbits against the peptide sequence NH$_{2}$-(C)T$^{437}$VPKDPEAAEARRG$^{445}$-COOH spanning the ADAMTS cleavage site (in italics) in the versican-V1 core protein. The N-terminal Cys residue was added for conjugation to keyhole limpet hemocyanin, and the keyhole limpet hemocyanin-peptide conjugate was injected into rabbits (YenZym Antibodies, LLC, South San Francisco, CA). Immune sera were affinity-purified against the immobilized peptide antigen. To block ADAMTS5 cleavage of versican V5-GAG, anti-VC was incubated with V-5GAG at increasing concentrations for 30 min at 37 °C. These V-5GAG-anti-VC complexes were then used in subsequent versican digestion (versicanase) assays.

Characterization of Anti-DPEAAE Specificity—NH$_{2}$-DPEAAE-COOH peptide or variations of it were synthesized by the Lerner Research Institute Molecular Biotechnology Core. Versikine-containing conditioned medium was diluted 1:2 in coating buffer (40 mM Na$_{2}$CO$_{3}$ (pH 9.6)), and 200 μl was used to
coat F96 Maxisorb plates (Nunc, Rochester, NY) by overnight incubation at room temperature. The wells were washed with 50 mM HEPES, 100 mM NaCl, 0.05% (v/v) Tween 20 (pH 7.4), blocked by incubating with 200 µl of 1% (w/v) BSA (2 h, 37 °C), and the washing steps were repeated. Anti-DPEAAE (Affinity Bioreagents, Golden, CO) was preincubated with increasing concentrations of the peptides for 30 min at 37 °C. These were incubated with the versikine-coated wells (4 h, 37 °C) and washed. Alkaline phosphatase-conjugated rabbit antibody (Bio-Rad) was added to each well (2 h, 37 °C) and detected using p-nitrophenyl phosphate tablets (Sigma) and detection of the product at A_{405}.

Quantification of ADAMTS5 Concentration—ADAMTS5 concentration in the single batch of HEK293F conditioned medium used for this study was determined using a solid phase binding assay. Purified, recombinant ADAMTS5 Pro-Cat-Dis (provided by Dr. David Buttle, Sheffield University, UK) was coated overnight (in coating buffer) on F96 Maxisorb plates at increasing concentrations alongside multiple dilutions of ADAMTS5 conditioned medium. The wells were washed and blocked as described above, and ADAMTS5 was detected using 12F4, a monoclonal antibody with a conformational epitope spanning the catalytic and disintegrin-like domains (GlaxoSmithKline, King of Prussia, PA).3 Anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad) was added, and the bound antibody was detected using p-nitrophenyl phosphate tablets. Nonspecific antibody interactions were accounted for by subtracting the absorbance of wells coated with empty vector control conditioned medium. The concentration of ADAMTS5 was deduced from the monoclonal antibody-binding curve generated from the absorbance at 405 nm of the recombinant protein. This gave a value of ~3 µg/ml. For all versican digests, 100 µl of conditioned medium was used (~300 ng of ADAMTS5).

Versican Antibodies, Versican Digestion, Western Blotting, and Densitometry—To detect versicanase activity, conditioned medium from ADAMTS transfections was combined with versican conditioned medium in a 1:1 ratio, incubated for 16 h at 37 °C, and analyzed using 10% SDS-PAGE. Alternatively, HEK293F cells were cotransfected with 1 µg each of ADAMTS5 and versican plasmids, and the conditioned medium was analyzed as above. Western blotting was done under reducing conditions using anti-VC or anti-DPEAAE; anti-GAG3 (against amino acids 1028–1274), anti-GAG4 (against amino acids 1659–2101), and anti-GAG5 (against amino acids 357–567) (14), anti-GAG12F4, a monoclonal antibody with a conformational epitope spanning the catalytic and disintegrin-like domains (Glaxo-SmithKline, King of Prussia, PA).3 Anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad) was added, and the bound antibody was detected using p-nitrophenyl phosphate tablets. Nonspecific antibody interactions were accounted for by subtracting the absorbance of wells coated with empty vector control conditioned medium. The concentration of ADAMTS5 was deduced from the monoclonal antibody-binding curve generated from the absorbance at 405 nm of the recombinant protein. This gave a value of ~3 µg/ml. For all versican digests, 100 µl of conditioned medium was used (~300 ng of ADAMTS5).

Synthesis of Click-xyloside and Xyloside Treatment of Cells—Click-xyloside synthesis was as described previously (52). Briefly, copper-catalyzed click chemistry was performed at room temperature in 1:1 acetonewater. 1.2 molar equivalents of pentyne (catalog no. Wako 322-49451, Wako Chemicals, Richmond, VA), 0.2 mol equivalents of aqueous CuSO4, and 0.4 mol equivalents of l-sodium ascorbate were added to 1 molar equivalent peracetylated β-xylosyl azide. The reaction product, click-xyloside, was purified on a flash silica column using an ethyl acetate-hexane gradient. The purified product was subsequently deprotected in dry MeOH/sodium methoxide at pH 10. The deprotected compound was purified on a reverse phase silica column to obtain the final product, MQ-1-31, which was characterized by 1H NMR and negative mode LC-MS.

HEK-293F cells stably expressing V-5GAG were seeded to 30% confluence and incubated for 16 h in medium supplemented with 10% PBS and antibiotics. The cells were washed with PBS, and the medium was replaced with serum-free medium containing click-xyloside (dissolved in dimethyl sulfoxide) at a stock concentration of 10 mM) at the appropriate concentration. For controls, the appropriate amount of dimethyl sulfoxide was added to the medium. After 48 h of further culture, the conditioned medium was collected, and the effect of increasing click-xyloside concentration on V-5GAG cleavage by ADAMTS5 was determined.

V-5GAG Pulldown by the ADAMTS5 Ancillary Domain—Anti-FLAG-agarose beads (50 µl) were washed three times with TBS, added to conditioned medium containing the ADAMTS5 ancillary domain, and incubated at room temperature for 2 h. The resin was washed five times with TBS (150 mM NaCl and 50 mM Tris-HCl (pH 7.6)), added to V-5GAG conditioned medium, and incubated at room temperature for a further 2 h. The resin was washed five times with TBS and resuspended to a volume of 100 µl with TBS prior to digestion with chondroitinase ABC (0.1 units/ml) at 37 °C for 2 h. The supernatant was analyzed by Western blotting for V-5GAG using anti-VC. A control pulldown assay was performed using the medium of cells transfected with an empty vector (p3XFLAG-CMV9) instead of the ADAMTS5 ancillary domain conditioned medium. The purpose of this control was to show that V-5GAG was not binding nonspecifically to the anti-FLAG-agarose beads.

Particle Exclusion Assay—The RBC exclusion assay was used to visualize the pericellular matrix and was carried out essentially as described previously (10). Briefly, formalin-fixed sheep RBCs were washed with PBS and resuspended to a final concentration of 1.0 × 10^6 RBCs/ml. Dermal fibroblasts obtained
from wild-type C57Bl/6J mice were plated at ~30% confluence in 6-well plates and incubated in serum-free medium with anti-VC or control rabbit isotype-matched IgG antibody for 24 h. The RBC suspension (200 μl) was added to each well along with calcein (final concentration 1 μg/ml) for cell visualization and incubated for 20 min to allow the RBCs to settle around the cells. Images of the cells were taken with an inverted wide-field Leica microscope (DR IRB, Heidelberg, Germany) using a ×20 objective lens in fluorescent and phase-contrast modes. Pericellular matrix exclusion zones were quantified using ImageJ (Media Cybernetics, Silver Spring, MD) by subtracting the area of the fluorescent image (i.e. the cell) from the total area of the cell plus the exclusion zone as observed in phase-contrast mode.

Collagen Gel Contraction Assay—The collagen gel contraction assay was performed as described previously (10). Melted 4% agarose (Amresco, Solon, OH) was allowed to gel in 24-well plates around 10-mm cloning rings to form 10-mm diameter molds for the collagen gels. Rat tail collagen (3.2 mg/ml, catalog no. 354236, BD Biosciences) was diluted to a final concentration of 1.6 mg/ml with DMEM containing 10% FBS, antibiotics, and dermal fibroblasts (2 × 10⁵ cells). Antibody (either anti-VC or rabbit IgG isotype-matched control) was added at the appropriate concentration, and the gels were allowed to polymerize at 37 °C for 1 h. The gels were overlaid with 1 ml DMEM supplemented with 10% FBS, antibiotics, and either anti-VC or the control antibody at the appropriate concentration. The gels were detached from the agarose mold and allowed to contract overnight (16 h) at 37 °C as suspended gels. The gels were visualized under a stereomicroscope, and the area was quantified using ImageJ.

Statistical Analysis—Data represent the mean ± S.D. of at least three independent experiments. Statistical analysis was performed using the unpaired Student’s t test.

RESULTS

The Glu⁴⁴¹-Ala⁴⁴² Bond Is a Major Site of Versican Proteolysis in the Versican GAGβ Domain—New human versican-V1 constructs (Fig. 1A) and a new versican antibody to a peptide straddling the Glu⁴⁴¹-Ala⁴⁴² bond, named anti-VC, were gen-

FIGURE 1. Characterization of versican constructs used for this analysis. A, domain composition of constructs showing the globular domains G1 and G3 and the CS-bearing GAGβ domain specific for versican-V1. The peptide used as the immunogen for anti-VC is shown. The arrows indicate the ADAMTS-targeted Glu⁴⁴¹-Ala⁴⁴² peptide bond. B, Western blot analysis with the indicated antibodies following reducing SDS-PAGE showing versican-V1 expression in conditioned medium and characteristically improved detection after digestion with chondroitinase ABC (C, ABC). C, detection of V-5GAG expression by the indicated antibodies. In B and C, chondroitinase ABC was necessary to resolve the proteoglycan as a sharper band and allow it to migrate fully into the resolving gel. D, ADAMTS cleavage of versican-V1 and V-5GAG detected using anti-VC. Bands relating to versikine or intact proteoglycan are indicated with one or two asterisks, respectively.

from wild-type C57Bl/6J mice were plated at ~30% confluence in 6-well plates and incubated in serum-free medium with anti-VC or control rabbit isotype-matched IgG antibody for 24 h. The RBC suspension (200 μl) was added to each well along with calcein (final concentration 1 μg/ml) for cell visualization and incubated for 20 min to allow the RBCs to settle around the cells. Images of the cells were taken with an inverted wide-field Leica microscope (DR IRB, Heidelberg, Germany) using a ×20 objective lens in fluorescent and phase-contrast modes. Pericellular matrix exclusion zones were quantified using ImageJ (Media Cybernetics, Silver Spring, MD) by subtracting the area of the fluorescent image (i.e. the cell) from the total area of the cell plus the exclusion zone as observed in phase-contrast mode.

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RESULTS

The Glu⁴⁴¹-Ala⁴⁴² Bond Is a Major Site of Versican Proteolysis in the Versican GAGβ Domain—New human versican-V1 constructs (Fig. 1A) and a new versican antibody to a peptide straddling the Glu⁴⁴¹-Ala⁴⁴² bond, named anti-VC, were gen-
Western blotting of full-length versican (V1) and V-5GAG transfected into HEK293F cells demonstrated that they were modified appropriately with CS chains because a high molecular weight smear arising from each was resolved into a sharper band of stronger intensity following chondroitinase ABC digestion (Fig. 1, B and C). Anti-VC detected versican-V1 and V-5GAG specifically after digestion with chondroitinase ABC, with a reactivity similar to commercial anti-GAG/H9252 and anti-myc antibody on Western blot analyses (Fig. 1, B and C). The specificity of anti-VC was validated by blockade of its reactivity against V-5GAG on Western blot analyses after incubation with the peptide immunogen (data not shown). Without chondroitinase ABC digestion, as expected, versican-V1 and V-5GAG migrated poorly into the gel or not at all (Fig. 1, B and C). V-5GAG, which contains fewer CS chains than V1 and is smaller, was detectable using anti-myc but not anti-VC (Fig. 1C). The observed difference likely results from differences between the affinities and optimal concentrations of the two antibodies in these Western blot analyses. V-5GAG, but not V1, was detectable by anti-myc on Western blot analyses (Fig. 1C and data not shown for V1), possibly because of proteolytic loss of the tag in the latter construct. In subsequent experiments, we used anti-GAGβ for detection of V1, anti-myc for detection of V-5GAG, and anti-VC for detection of either construct. When either versican-V1 or V-5GAG were digested with ADAMTS5-containing medium, but not the medium of vector-transfected cells, and the digests were

![Graph](image-url)

**FIGURE 2.** Characterization of anti-DPEAAE and recombinant human versikine. A, competitive solid-phase binding assay examining the reactivity of anti-DPEAAE. Versikine was coated in each well and detected by anti-DPEAAE antibody preincubated with the indicated peptides at increasing concentrations (data points are shown as mean ± S.E., n = 6). Peptides other than DPEAAE do not block anti-DPEAAE effectively. B, representative Western blot analyses contrasting versikine and versikine-myc immunoreactivity to anti-VC (left panel) and anti-DPEAAE (right panel). Anti-DPEAAE fails to detect versikine-myc as a consequence of epitope masking by the myc tag. IB, immunoblot. C, representative Western blot analysis showing the change in migration of versikine-myc following peptide N-glycanase F (PNGase F) treatment. D, representative Western blot analysis demonstrating that ADAMTS5 does not cleave versikine. Versikine (left panel) and versikine-myc (right panel) were incubated with ADAMTS5 or empty vector control conditioned medium and probed with anti-VC. No change in molecular species was observed following ADAMTS5 treatment.
treated with chondroitinase ABC, a 70-kDa band corresponding to versikine was obtained (Fig. 1D). ADAMTS5 cleaved versican upon coincubation of a versican construct with ADAMTS5 conditioned medium as well as after cotransfection of ADAMTS5 and versican constructs.

We evaluated the specific immunoreactivity of anti-DPEAAE to versikine by preincubating the antibody with a variety of peptides, such as those that deleted Glu441 (DPEAA), replaced it with Ala (DPEAAA), or added one or two C-terminal Ala residues (DPEAAEA, DPEAAEAA). Neither of these peptides blocked anti-DPEAAE reactivity against versikine as effectively as peptide DPEAAE (Fig. 2A). Anti-DPEAAE failed to react with versikine when a C-terminal myc-His, tag was present (Fig. 2B). In contrast, anti-VC could detect versikine or versikine-myc-His, with similar reactivity on Western blot analyses. Therefore, anti-DPEAAE is a true neoeptope antibody to versikine that is absolutely dependent on Glu441 for its reactivity, whereas anti-VC detects versikine because it contains 10 of the 14 immunogen peptide residues (Fig. 1A). As shown in Fig. 1, anti-VC can detect versikine in samples electrophoresed without prior chondroitinase ABC digestion as well as intact versican substrate if the sample is digested prior to electrophoresis (Fig. 1, B–D). In subsequent experiments, we used anti-DPEAAE or anti-VC to detect versikine but did not use anti-DPEAAE for analysis of cleavage site mutants because of its stringent specificity.

Consistent with previous reports, versikine migrated electrophoretically with an observed molecular mass of ~70 kDa (Figs. 1D and 2, B–D), which was inconsistent with its predicted mass of 48.9 kDa. Because versikine lacks CS chains, we digested it with peptide N-glycanase F to determine whether the discrepancy could be explained by modification at three potential sites for N-glycosylation, i.e. Asn57, Asn330, and Asn411. When treated with peptide N-glycanase F, the observed molecular mass was reduced by ~5 kDa (Fig. 2C). The presence of a highly negatively charged region (amino acids 361–408) in versikine likely leads to local intrinsic disorder that can manifest as aberrant migration in SDS-PAGE. Indeed, analysis of the sequence of versikine using online prediction tools (IUPreD and FoldIndex) predicted a strong tendency to local disorder in residues 360–441. When versikine or myc-tagged versikine were digested with ADAMTS5 and the digests were immunoblotted with anti-VC, versikine migration was unchanged, suggesting that, when released from versican, versikine was not cleaved further by ADAMTS5 (Fig. 2D).

To investigate whether cleavage occurred at additional sites within the GAGβ domain, we analyzed ADAMTS5-digested versican-V1 by Western blotting with four polyclonal antibodies spanning the GAGβ-domain (Fig. 3A). Antibody A, the most N-terminal and adjacent to the G1 domain, detected a 70-kDa band similar to anti-VC when either V1 or V-5GAG were incubated with ADAMTS5 (Fig. 3B). This species likely corresponds

FIGURE 3. GAGβ cleavage by ADAMTS5 occurs at the Glu441-Ala442 site. A, locations of peptides used as immunogens for a series of rabbit polyclonal rabbit antiseras GAGβ A-D are indicated. The arrow shows the location of the Glu441-Ala442 cleavage site. B, Western blot analyses of ADAMTS5 digests of versican-V1 (using antibodies GAGβA-GAGβD) or V-5GAG (using antibodies GAGβA and GAGβB) following chondroitinase ABC digestion and reducing SDS-PAGE. Conditioned medium obtained from empty vector-transfected cells was the control. The expected locations of versican-V1, V-5GAG, and versikine are indicated. IB, immunoblot.
to versikine because the peptide used to generate antibody A, like the VC peptide, spans the Glu441-Ala442 processing site. However, antibodies B-D did not identify fragments resulting specifically from digestion with ADAMTS5 (Fig. 3B). We conclude that ADAMTS5 did not process the versican GAG core protein at sites other than Glu441-Ala442. However, fragments not resulting from ADAMTS5 digestion (i.e. observed in both the experimental and control lanes) were seen, suggesting that versican may be cleaved by other proteases expressed by HEK293F cells (Fig. 3B).

The ADAMTS5 Ancillary Domain Binds to Versican-V1 and Is Essential for Proteolysis—To determine which region of ADAMTS5 bound to versican, we utilized a construct containing the propeptide, catalytic domain, and disintegrin-like domain (ADAMTS5 Pro-Cat-Dis) or the entire ancillary domain (Fig. 4A). In contrast to full-length ADAMTS5, ADAMTS5 Pro-Cat-Dis did not cleave versican V5-GAG, suggesting a requirement of the ancillary domain for versican binding (Fig. 4B). To investigate whether the ancillary domain promoted versican cleavage by localizing ADAMTS5 to the versican core protein, coimmunoprecipitation was performed. The FLAG epitope-tagged ADAMTS5 ancillary domain was first successfully pulled down using anti-FLAG-agarose beads (Fig. 4C). The FLAG resin + ancillary domain complex was incubated with V-5GAG conditioned medium and washed extensively. The FLAG resin + ancillary domain complexes were incubated with chondroitinase ABC, and the supernatant was analyzed by Western blotting using anti-VC. A coprecipitating band of ~150 kDa corresponding to V-5GAG was observed (Fig. 4D). This band was not seen when immunoprecipitation was performed using empty vector-transfected conditioned medium as a control. Therefore, the ancillary domain of ADAMTS5 interacted specifically with V-5GAG via the CS chains.

Versican Chondroitin Sulfate Chains Are Required for Proteolysis by ADAMTS5—When either versican-V1 or V-5GAG were digested with chondroitinase ABC prior to incubation with ADAMTS5, there was a substantial reduction in band intensity of versikine (Fig. 5A), suggesting that CS chains play a central role in mediating ADAMTS5 proteolysis at the Glu441-Ala442 site. Because V-5GAG digestion by ADAMTS5 gave a comparable versikine product, as did a digest of versican-V1 (Figs. 1D and 5A), we considered it likely that V-5GAG contained the determinant(s) necessary for processing at Glu441-Ala442. We compared proteolysis of V-5GAG expressed in
CHO-K1 cells and the mutant derivative cell line, CHO-K1 pgs-745A, which lacks xylosyltransferase and is, therefore, unable to add GAG chains to core proteins. ADAMTS5 did not efficiently cleave V-5GAG expressed from CHO-K1 pgs745A cells, despite comparable levels of core protein secreted from CHO-K1 or CHO-K1 pgs745A cells (Fig. 5B). In addition, digests of V-5GAG from CHO cells cultured in the presence of a click-xyloside, which reduces GAG-attachment to core proteins, demonstrated a dose-dependent reduction of versikine production relative to the amount of V5-GAG secreted by the cells (Fig. 5C). Together, these results clearly indicate a key role for the CS chains in mediating ADAMTS5 cleavage of versican.

To determine whether specific CS chains of V-5GAG mediated ADAMTS5 cleavage, we mutated four of the CS attachment sites (Fig. 6A). Loss of individual GAG attachment sites did not affect the secretion efficiency of the respective mutants, as evident from comparable levels of each mutant in the medium of transfected cells (Fig. 6B, bottom panel). However, loss of two CS chains nearest the Glu441-Ala442 scissile bond (i.e. mutagenesis of CHO-K1 cells and the mutant derivative cell line, CHO-K1 pgs-745A, which lacks xylosyltransferase and is, therefore, unable to add GAG chains to core proteins. ADAMTS5 did not efficiently cleave V-5GAG expressed from CHO-K1 pgs745A cells, despite comparable levels of core protein secreted from CHO-K1 or CHO-K1 pgs745A cells (Fig. 5B). In addition, digests of V-5GAG from CHO cells cultured in the presence of a click-xyloside, which reduces GAG-attachment to core proteins, demonstrated a dose-dependent reduction of versikine production relative to the amount of V5-GAG secreted by the cells (Fig. 5C). Together, these results clearly indicate a key role for the CS chains in mediating ADAMTS5 cleavage of versican.

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Ser507 and Ser525) led to a statistically significant reduction in versikine product. Elimination of CS attachment to Ser644 or Ser646 (by mutating Gly645) and Ser655 was without a similar effect (Fig. 6B). Therefore, we conclude that the two most N-terminal CS chains are required for processing by ADAMTS5. To test this possibility, versicanase digests were undertaken using V-2GAG, a construct containing only the two N-terminal CS attachment sites identified as crucial after mutagenesis of V5-GAG, i.e. Ser507 and Ser525 (Fig. 6C). This construct was cleaved efficiently by ADAMTS5 (Fig. 6C). We conclude that the CS chains attached to Ser507 and Ser525 are necessary and sufficient for versican-V1 processing by ADAMTS5.

FIGURE 6. The two N-terminal most CS chains are essential for versican proteolysis at the Glu441-Ala442 site. A, schematic depicting the mutants used in this analysis. The locations of the individual mutations that abolish CS attachment are shown by the dotted line. Solid lines indicate CS chain attachment sites that were not mutated in the respective constructs. B, representative Western blot analysis of V-5GAG mutants after digestion with ADAMTS5. Note the reduction of versikine specifically in the Ser507 and Ser525 mutants. Top panel, bottom, Western blot analysis with anti-myc illustrating the amounts of each mutant that were used in the analysis and that the mutations did not substantially affect secretion. For detection of V5-GAG by anti-myc, samples were enzymatically deglycosylated with chondroitinase ABC prior to electrophoresis. Bottom panel, quantitative analysis of Western blot analyses from three independent experiments. *, p < 0.05. C, Western blot analysis of the V-2GAG construct (top panel) digested with ADAMTS5 or vector-conditioned medium as a control for ADAMTS5. V2-GAG was modified by CS attachment (bottom left panel) and underwent efficient digestion by ADAMTS5 to generate versikine (bottom right panel). C’ ABC, chondroitinase ABC.
Recently, a novel versican isoform, V4, which arises by use of a cryptic splice site in the GAG/H9252-encoding exon, was described. V4 contains G1, G3, and the five N-terminal CS-attachment sites. It is essentially similar to the V-5GAG construct other than having a C-terminal G3 domain. A V4 construct was processed by ADAMTS5 comparably with V-5GAG (data not shown).

**Glu441 Is Required for Versican Proteolysis by ADAMTS5**—In view of the prevalence of Glu as the P1 residue in peptide bonds cleaved by ADAMTS proteases in aggrecan and versican (35, 39), we asked whether proteolysis of V-5GAG was affected when Glu441 was mutated (to Ala) (Fig. 7A). This mutant was secreted into medium of transfected cells at comparable levels as V-5GAG (Fig. 7B), but its digestion by ADAMTS5 was reduced substantially (Fig. 7C). Instead, an anti-VC reactive fragment was observed that migrated slightly more rapidly, suggestive of proteolysis at a site immediately upstream, i.e. following Glu 438. When both Glu438 and Glu441 were mutated, however, no digestion of V-5GAG occurred, as detected by both anti-VC and an antibody to the versican G1 domain (Fig. 7C). Notably, these mutations did not abolish versican recognition by anti-VC (Fig. 7B, compare top and bottom panels).

**Anti-VC Blocks Versican Processing**—Because the anti-VC immunogen peptide straddles the Glu441-Ala442 cleavage site, we asked whether anti-VC antibody binding to versican could sterically hinder its proteolysis. Incubation of V-5GAG with anti-VC demonstrated a dose-dependent accumulation of undigested V-5GAG (Fig. 8A) and reduced the versikine product in the digests (Fig. 8B), indicative of inhibition of versican proteolysis. Previously, loss of ADAMTS5 activity in skin fibroblasts has been shown to lead to an accumulation of a versican-rich pericellular matrix and a fibroblast-to-myofibroblast transition (10). When wild-type mouse skin fibroblasts were treated with anti-VC, there was an accumulation of pericellular matrix (Fig. 9A) and enhanced contractility of dermal fibroblasts in collagen gels (Fig. 9B). This effect of anti-VC, similar to that demonstrated previously upon inactivation of ADAMTS5 or overexpression of versican-V1 (10), suggests that anti-VC can be used to block versican proteolysis by ADAMTS5.

**ADAMTS1 Has Similar Requirements for Versican Processing as ADAMTS5**—ADAMTS1 proteolysis of versican is required for ovulation (53, 54) and for compaction of the developing myocardium (55). Here we extend the major findings of our investigation to ask whether ADAMTS1 employed similar mechanisms as ADAMTS5 for versican processing. Like ADAMTS5, ADAMTS1 could generate versikine when V-5GAG was generated in CHO-K1 cells but not in xylosyl-
transferase-deficient pgs-745A cells (Fig. 10A). Furthermore, mutation of Glu 441 and Glu 438 abrogated versican processing by ADAMTS1 (Fig. 10B), and prevention of GAG attachment at Ser 507 and Ser 525 by mutagenesis of these residues also prevented or reduced proteolysis by ADAMTS1 (Fig. 10C).

**DISCUSSION**

Because of the great interest in characterizing ADAMTS4 and ADAMTS5 activity in osteoarthritis, proteolysis of aggrecan, the major cartilage proteoglycan, has been investigated extensively (39, 40). However, the mechanisms of versican cleavage have not been investigated previously. This analysis, focusing on ADAMTS5 and ADAMTS1, which are major versicanases during embryogenesis, demonstrates similarities and distinctions between ADAMTS proteolysis of versican and aggrecan. These studies show that, unlike aggrecan, which is cleaved by ADAMTS proteases, including ADAMTS5, at multiple sites within the CS-bearing domain (39, 40), cleavage of the versican-V1 core protein primarily occurred at the Glu 441-Ala 442 site or, in its absence, at a putative upstream site but not elsewhere within the GAG-domain.

We have shown here that ADAMTS5 relies on specific determinants in versican for interaction and proteolysis respectively, i.e. two specific CS chains and Glu 441. These findings constitute the first understanding of how versican is cleaved by an ADAMTS protease. Initially, the analysis showed that enzymatically eliminating CS modification of the core protein led to reduced proteolysis. Because digestion by chondroitinase ABC leaves residual core oligosaccharide stubs, we sought additional evidence using CHO-pgs745A cells, which lack the ability to attach xylose (51), the first residue of the nascent CS chain. Reduced ADAMTS5 and ADAMTS1 proteolysis of V-5GAG expressed by these cells or by cells cultured in the presence of a click-xyloside that acts as a decoy for CS attachment (52) supported the requirement of CS chains in V-5GAG for proteolysis. We conclude that the two N-terminal-most CS chains are likely binding sites for ADAMTS5 exosites and provide it with access to the Glu 441-Ala 442 site. ADAMTS5 binding to the CS chains could lead to a conformational change in the versican core protein that renders the Glu 441-Ala 442 site accessible or contributes to opening of the ADAMTS5 catalytic site, previously shown to exist in both open and closed conformations (56).

Previously, an Escherichia coli-expressed, GAG-free versican polypeptide spanning residues Gly 357 to Asp 567 has been used to demonstrate proteolytic processing of versican by ADAMTS1 and ADAMTS4 (35). In contrast, our work suggests that CS-modified versican is the preferred ADAMTS5 and ADAMTS1 substrate. We speculate that the CS-chains provide an anchorage site near the scissile bond that may be otherwise elusive in a polypeptide that is predicted to be unstructured. At the scissile bond, Glu 441 was an essential determinant, and its elimi-
FIGURE 9. Anti-VC induces the accumulation of pericellular matrix and myofibroblast transition in mouse dermal fibroblasts. A, erythrocyte exclusion assay demonstrating increased pericellular matrix around dermal fibroblasts in the presence of anti-VC. Arrows indicate the pericellular matrix border around calcein-labeled fibroblasts (green). Representative images are shown for both control IgG and anti-VC. Quantification of pericellular matrix area demonstrates a significant increase in anti-VC treated cells ($p < 0.005$), as shown in the whisker plot (bottom panel), attributed to reduction in versican cleavage. Each dot in the plot is a single cell measurement. B, collagen contraction assay to evaluate fibroblast contractility in the presence of anti-VC. Representative images are shown in the top panel. Quantification of the gel area demonstrates a significant reduction in the presence of anti-VC ($p < 0.005$), suggestive of increased contraction of the collagen gel by embedded fibroblasts (bottom panel). Each dot in the plot represents a single gel.

FIGURE 10. ADAMTS1 cleavage of V-5GAG uses similar molecular determinants as ADAMTS5. A, versican and V-5GAG were expressed in CHO-K1 cells or the pgs745A CHO-K1 mutant (as shown in Fig. 5) and incubated with conditioned medium containing ADAMTS1. A reduction in versikine generation was observed using V-5GAG expressed in pgs745A CHO-K1 cells. C'ABC, chondroitinase ABC; IB, immunoblot. B, Western blot analysis showing the effect of the cleavage site mutants (described in Fig. 7) on digestion by ADAMTS1. Cleavage, speculated to occur after Glu$^{438}$, is observed in mutant A (indicated by the asterisk). Cleavage at this site is eliminated following mutation of Glu$^{438}$ (V-5GAGB). C, representative Western blot analysis of V-5GAG CS chain attachment mutants (described in Fig. 6) after digestion with ADAMTS1 followed by chondroitinase ABC treatment. The two CS chain attachment mutants that had reduced cleavage by ADAMTS5 also had reduced ADAMTS1-mediated versican cleavage (versikine is indicated by the asterisk). A chondroitinase ABC digest of the V-5GAG conditioned medium shows comparable expression levels of all constructs.
in a site immediately upstream, which we posit to be the Glu\textsuperscript{438}-Ala\textsuperscript{439} bond. The precise site could not be determined because of the low prevalence of the alternative cleaved fragment and the inherent difficulty of C-terminal protein sequencing.

ADAMTS4 and ADAMTS5 cleave aggrecan not only within the interglobular domain but also at several other sites within the GAG-bearing region (39, 40, 57). Aggrecan has a higher density of GAG attachment sites than versican, and the GAG attachment region is divided into an N-terminal CS1 and a C-terminal CS2 domain (58). Addition of glycosaminoglycan sugars to articular explant cultures inhibited ADAMTS1, 4, and 5 activities, presumably by competing with the GAGs on aggregan (59).

Previous work examining ADAMTS4 cleavage of aggrecan had not identified specific CS chains as crucial determinants of proteolysis within the CS2 region (60, 61). However, the requirement for the ancillary domain has been established for ADAMTS4 and ADAMTS5 cleavage of aggrecan (62, 63) and appears to be similar for cleavage of versican. Therefore, the ADAMTS5 ancillary domain, on the basis of its binding to the CS chains, likely contains one or more exosites for ADAMTS5 activity against aggrecan and versican. Such exosites are necessary because the catalytic domains of ADAMTS proteases typically have little activity against native substrates, and most binding attributes are located in the ancillary domain. For example, ADAMTS1 binding to the extracellular matrix was dependent on its ancillary domain (64), TSR1 of ADAMTS4 has been shown to be essential for aggrecanase activity through binding to the CS chains (61), and truncation experiments as well as chimeric proteins of ADAMTS4 and ADAMTS5 have shown altered activity against aggrecan after manipulation of the ancillary domains (63, 65). Furthermore, the crystal structure of the ADAMTS13 ancillary domain in conjunction with mutagenesis suggested the presence of several discontinuous exosites for its substrate von Willebrand factor (66). The importance of exosites in substrate recognition is particularly evident in thrombotic thrombocytopenic purpura, where autoantibodies are commonly directed to ADAMTS13 exosites, inhibiting substrate recognition and reducing von Willebrand factor processing (67, 68).

Anti-DPEAAE antibody is a widely used and valuable tool for versican analysis (10, 16, 17, 33, 35, 42, 44–46, 53, 56–58), but the determinants of its reactivity have not been mapped previously. Our finding that Glu\textsuperscript{441} was absolutely required for anti-DPEAAE reactivity necessitated the development of an antibody for this work whose reactivity was not dependent on the presence of Glu\textsuperscript{441}. As shown here, anti-VC retains its reactivity and specificity after mutagenesis of both Glu\textsuperscript{441} and Glu\textsuperscript{438}. Furthermore, we have shown that anti-VC is function-blocking for proteolysis by ADAMTS5 and can be used to manipulate versican processing \textit{in vitro} and, potentially, \textit{in vivo}. Applied to skin fibroblasts, anti-VC has effects similar to those elicited previously by genetic inactivation of ADAMTS5, i.e., accumulation of pericellular matrix and a fibroblast-to-myofibroblast phenotype switch demonstrated by enhanced contraction of a collagen gel (10).

Previous work provided both genetic and biochemical evidence strongly implicating versican proteolysis at the Glu\textsuperscript{441}-Ala\textsuperscript{442} site as a major mechanism underlying several ADAMTS mouse mutant phenotypes. However, the conclusion that versican is their principal target can be unequivocally made only if it can be shown that rendering versican uncleavable leads to similar phenotypes. This work provides information that will be useful for resolution of this question. We identify three potentially useful approaches for preventing versican processing, i.e., elimination of GAG attachment at Ser\textsuperscript{507} and Ser\textsuperscript{525}, replacement of Glu\textsuperscript{441} by Ala, or administration of anti-VC blocking antibody. Mouse models incorporating mutations at these sites could be useful for rigorous evaluation of versican as the principal \textit{Adams1}, \textit{Adams5}, \textit{Adams20}, and \textit{Adams9} substrate in cleft palate, soft tissue syndactyly, white spotting of skin, and myocardial and valvular development.

This work provides a proof of principle for preventing cleavage of versican, aggrecan, and brevican by steric hindrance of ADAMTS proteases using antibodies to the region of their scissile bonds. Such an approach is potentially of therapeutic interest in osteoarthritis for prevention of aggrecan proteolysis at select sites and in gliomas for preventing cell migration and invasion induced by ADAMTS-processed brevican (69, 70). Indeed, for proteins cleaved by multiple proteases, selective targeting of a cleavage site in the manner demonstrated here may be both more effective and less prone to side effects than protease blockade.

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