Fibulin-1 Acts as a Cofactor for the Matrix Metalloprotease ADAMTS-1*

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ADAMTS-1 is a metalloprotease that has been implicated in the inhibition of angiogenesis and is a mediator of proteolytic cleavage of the hyaluronan binding proteoglycans, aggrecan and versican. In an attempt to further understand the biological function of ADAMTS-1, a yeast two-hybrid screen was performed using the carboxyl-terminal region of ADAMTS-1 as bait. As a result, the extracellular matrix protein fibulin-1 was identified as a potential interacting molecule. Through a series of analyses that included ligand affinity chromatography, co-immunoprecipitation, pull-down assays, and enzyme-linked immunosorbent assay, the ability of these two proteins to interact was substantiated. Additional studies showed that ADAMTS-1 and fibulin-1 colocalized in vivo. Furthermore, fibulin-1 was found to enhance the capacity of ADAMTS-1 to cleave aggrecan, a proteoglycan known to bind to fibulin-1. We confirmed that fibulin-1 was not a proteolytic substrate for ADAMTS-1. Together, these findings indicate that fibulin-1 is a new regulator of ADAMTS-1-mediated proteoglycan proteolysis and thus may play an important role in proteoglycan turnover in tissues where there is overlapping expression.

Proteolytic events within the extracellular matrix (ECM)‡ are essential for developmental morphogenesis, homeostasis of adult tissues, and pathological conditions such as wound healing and tumor angiogenesis (1–3). ADAMTS-1 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs) belongs to a family of metalloproteases involved in the processing of ECM proteins such as procollagen types I and II (4, 5), von Willebrand factor (6–8), and proteoglycans, including aggrecan, versican, and brevican (9–12).

A large percentage of mice that lack ADAMTS-1 die perinatally (45%), and those that survive display urinary tract obstructions and kidney pathologies (13, 14). In addition, most ADAMTS-1 null females are infertile because of abnormalities in the ovaries and endometrium (13). Evaluation of secondary follicles in the ovaries of ADAMTS-1 null mice revealed poor cumulus expansion and defective ovulation. These anomalies were attributed to incomplete versican proteolysis (15).

Thus, a concrete understanding of the array of ADAMTS-1 biological functions will likely rely on insights from its substrates and regulation of catalytic function.

The fibulins are a family of secreted glycoproteins that are components of elastic matrix fibers and basement membranes. Currently, there are six members of the fibulin family (16). The prototypic member, fibulin-1, contains the signature structural features of all fibulin family members, a series of epidermal growth factor-like repeats followed by a fibulin-type module at its carboxyl terminus (16).

Functionally, fibulin-1 binds to many ECM proteins, including laminin, fibrinogen, fibronecrtin, nidogen-1, and endostatin, and to the proteoglycans, aggrecan and versican (17–22). Mice lacking fibulin-1 die perinatally and display vascular anomalies in the kidney in addition to extensive hemorrhage in several organs, likely related to abnormalities in endothelial cell interactions with subendothelial ECM (23).

Using a series of biochemical approaches, we have found that fibulin-1 binds to ADAMTS-1 and enhances its catalytic activities toward aggrecan. These findings, together with those showing that fibulin-1 can bind to aggrecan, led us to speculate that fibulin-1 acts to enhance ADAMTS-1-mediated proteolysis by facilitating a ternary complex formation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The Matchmaker Two-hybrid System 2 (Clontech Laboratories Inc. Palo Alto, CA) was used following the manufacturer’s recommendations. A cDNA fragment encoding amino acids 827–951 of human ADAMTS-1 (GenBank™ accession number AF060152) was amplified by PCR using the primers 5′-CACCTACT-TCTGATATGAAGAAG-3′ and 5′-CTTAAAACCTGACTG-CATTCTG-3′ and inserted into the vector pAS2–1 previously digested with NdeI and Sall restriction enzymes. The resulting construct encoded a GAL4BD-ADAMTS-1827–951 chimeric protein. A library of prey proteins from human placenta was obtained from Clontech Laboratories Inc. Both bait and prey vectors were simultaneously introduced into Saccharomyces cerevisiae CG-1945 cells and double transformants selected on synthetic minimal medium lacking tryptophan, leucine, and histidine. Colonies were restreaked and tested for β-galactosidase activity using a filter assay. DNA from positive colonies were amplified by PCR using Matchmaker 5′- and 3′-AD LD-Insert Screening Amplimers and the resulting amplicons sequenced.

Pulldown Assay—Fibulin-1 or BSA (5 µg each) were individually covalently linked to magnetic M450 tosyl-activated dynabeads (DYNAL, Brown Deer, WI) by overnight incubation in 100 mM phosphate buffer, pH 7.4, at 37 °C. The beads were subsequently washed with 200 mM Tris, pH 8.5, with 0.1% BSA and incubated overnight at 4 °C with ADAMTS-1 (2 µg) in a volume of 400 µl. Dynabeads were magnetically collected and washed with phosphate-buffered saline to...
removal of unbound proteins. Bound proteins were released using sample buffer containing β-mercaptoethanol and separated on 10% SDS-polyacrylamide gels, transferred to OptiTran nitrocellulose membrane (Schleicher and Schuell, Keene, NH), and immunoblotted with guinea pig anti-ADAMTS-1 polyclonal antibody (GPB).

**Co-immunoprecipitation Analysis**—Fragments of human full-term placenta were homogenized in cold lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM iodoacetamide, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 0.02% NaN₃, 1 mM PMSF, complete protease inhibitor mixture tablets following the manufacturer’s direction (Roche Applied Science), 1% Triton X-100 using a Waring blender. The lysate was incubated with protein G-agarose beads (Roche Applied Science) overnight at 4 °C to absorb resin-binding proteins. Aliquots of the lysates (1 ml) were then incubated with antibodies to fibulin-1 (mAb 3A11), ADAMTS-1 (GPB), or mouse IgG (Sigma) for 3 h at 4 °C. Antibody complexes were precipitated for 1 h at 4 °C using either protein G-agarose (Roche Applied Science) for mouse IgGs or protein A-agarose (Roche Applied Science) for GPB. The agarose-bound complexes were pelleted by centrifugation at 1,000 × g and the pellets washed with 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.5% Nonidet P-40. Bound proteins were released using sample buffer containing β-mercaptoethanol and separated on 10% SDS-polyacrylamide gels, transferred to OptiTran nitrocellulose membrane (Schleicher and Schuell), and analyzed by immunoblotting.

Conditioned medium from fibulin-1C-expressing HT1080 cells cultured in the absence of serum was collected and precleared with protein G-agarose beads (Roche Applied Science) for 16 h at 4 °C. Fibulin-1 monoclonal antibody (mAb 3A11) was added to 1-ml aliquots and incubated for 4 h at 4 °C. Immunocomplexes were pelleted with protein G-agarose beads and the pellets washed extensively using 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.5% Nonidet P-40 buffer. These fibulin-1-immobilized immunocomplexes were incubated with ADAMTS-1 (87-kDa form) for 2 h at 4 °C and unbound protein removed by washing with 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.5% Nonidet P-40 buffer. After several washes, bound proteins were released using sample buffer containing β-mercaptoethanol, separated on 10% SDS-polyacrylamide gels, and transferred to OptiTran nitrocellulose membrane (Schleicher and Schuell). The presence of ADAMTS-1 and fibulin-1 was evaluated by immunoblotting with GPB and mAb 3A11, respectively.

**Enzyme-linked Immunosorbent Assay**—Purified ADAMTS-1 (2.5 μg/ml) or fibulin-1 (10 μg/ml) in binding buffer (150 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 25 mM Tris, pH 8.2) was coated on Costar high binding 96-well plates for 16 h at 4 °C. Coated wells were incubated with 2% BSA in TBS with 0.5 mM CaCl₂, 0.5 mM MgCl₂ for 90 min at room temperature. Wells coated with ADAMTS-1 were incubated with fibulin-1 (10 μg/ml) in TBS with 0.5% BSA, 0.5 mM CaCl₂, and 0.5 mM MgCl₂ and vice versa for 90 min at room temperature. Plates were washed with TBS with 0.5% BSA, 0.5 mM CaCl₂, 0.5 mM MgCl₂. Bound proteins were detected in either mouse monoclonal antibodies against ADAMTS-1 (mAb 3E4/C6B4) or fibulin-1 (mAb 3A11), and anti-mouse-IgG-alkaline phosphatase conjugate. Colorimetric reactions were performed using p-nitrophenyl phosphate substrate (Sigma) and read at A = 405 nm using a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

**Solid Binding Assays**—Microwell plate wells were coated with human fibronectin, ADAMTS-1, or BSA at 3 μg/ml in TBS, pH 8.0, for 2 h at 37 °C and then rinsed three times with TBS, pH 7.4. Unbound sites were blocked for 1 h at room temperature with 5% nonfat milk in TBS, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.4. Wells were then rinsed three times with TBS, pH 7.4, and incubated with varying concentrations of fibulin-1 (ranging from 100 to 0.137 μg/ml) for 3–4 h at 37 °C in TBS containing 3% nonfat milk, 0.1% Tween 20, 1 mM CaCl₂, and 1 mM MgCl₂. After rinsing with TBS, 0.1% Tween 20 and 1 mM CaCl₂ and 1 mM MgCl₂, pH 7.4 (wash buffer), wells were incubated with anti-fibulin-1 monoclonal antibody (mAb 3A11) at 1 μg/ml in TBS, pH 7.4, containing 3% nonfat milk and 0.1% Tween 20, 1 mM CaCl₂, and 1 mM MgCl₂. Wells were then rinsed with wash buffer three times and incubated for 1 h at room temperature with anti-mouse horseradish peroxidase-conjugated IgG (1:5000 dilution, Amersham Biosciences) diluted in TBS, pH 7.4, containing 3% nonfat milk and 0.1% Tween 20. The wells were then rinsed three times with wash buffer, and detection was performed using TMB substrate (KPL, Gaithersburg, MD) with color development measured spectrophotometrically at λ = 650 nm. Curve fitting of data was performed using SigmaPlot 2001 software (SPSS Inc., Chicago, IL).

**ADAMTS-1 Affinity Chromatography**—ADAMTS-1 (2 mg) was coupled to 2 ml of CNBr-activated CL-4B-Sepharose (Amersham Biosciences) following the manufacturer’s instructions. Placental tissue (30 g) was extracted in 100 ml of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 1% Triton X-100, 0.02% NaN₃, 1 mM PMSF, 0.2 units/ml aprotinin, and 5 mM iodoacetamide, pH 8.0) using a Waring blender. The lysate was centrifuged at 3,000 × g for 30 min at 4 °C and then ultracentrifuged at 100,000 × g for 1 h at 4 °C. The lysate was precleared by passing through a 2-ml precolumn of glycine-Sepharose CL-4B (pre-equilibrated in lysis buffer) and loaded onto a 2-ml column of ADAMTS-1-Sepharose CL-4B (pre-equilibrated in lysis buffer) at a flow rate of 0.5 ml/min (Biologic Work station; Bio-Rad). The column was washed sequentially with 15 ml of lysis buffer and 15 ml of washing buffer (20 mM ethanolamine, 0.2% Triton X-100, 150 mM NaCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mM PMSF, pH 9). Bound proteins were eluted with a gradient starting at 20 mM ethanolamine, 0.2% n-octylglucoside, 150 mM NaCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mM PMSF, pH 9, and finishing at 20 mM ethanolamine, 0.2% n-octylglucoside, 1 M NaCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mM PMSF, pH 12. Fractions of 1 ml were collected and neutralized with 0.1 volume of 1 M Tris, pH 6.7. Eluted proteins were separated by SDS-PAGE and subjected to immunoblotting with anti-fibulin-1 (mAb 3A11).

**Analysis of ADAMTS-1, Fibulin-1, and Aggrecan Function in Embryonic Kidneys**—Kidneys harvested from E15.5 embryos were fixed in 2% paraformaldehyde overnight, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized and incubated with blocking buffer (BB, 5% goat serum and 2.5% BSA in PBS) for 2 h at room temperature. Sections were incubated with guinea pig anti-ADAMTS-1 (GP12) at 1:200 dilution in 0.5% BB, rabbit anti-fibulin-1 (Rb1323) (24) at 10 μg/ml in 0.5× BB, rabbit anti-aggrecan G1 domain or TASELE neo-epitope at 1:200 dilution in 0.5× BB for 2 h at room temperature. Sections were then incubated with anti-guinea pig IgG conjugated to biotin (Vector Labs, Burlingame, CA) at 1:250 dilution in 0.5× BB for 1.5 h, followed by incubation with streptavidin conjugated to CY3 (Sigma) at 1:100 dilution in 0.5× BB and anti-rabbit IgG conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch, West Grove, PA) at 1:150 dilution in 0.5× BB for 1 h at room temperature. Fluorescence immunohistochemistry was analyzed using a Bio-Rad MRC 1024ES laser confocal microscope.

Anti-ADAMTS-1 GP12 antibody was raised in guinea pig by BIO-SOURCE (Camarillo, CA) using purified ADAMTS-1 protein as immunizing antigen. Antibody specificity was tested against conditioned medium and cell lysates from T47D cells transfected with ADAMTS-1 or empty vector construct.

**Digestion of Fibulin-1 with ADAMTS-1**—Fibulin-1 (1 μg) was incubated with ADAMTS-1 at 1:1, 1:2, and 1:3 molar ratio in 50 mM Tris, pH 7.4, 10 mM CaCl₂, 80 mM NaCl overnight at 37 °C in a total volume of 1 ml.
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60 μl.Digests were separated in 10% SDSA-PAGE electrophoresis under reducing conditions using β-mercaptoethanol and followed by immunoblotting with antibodies to the amino terminus (mAb 3A11) or the carboxyl terminus (mAb 5D12) of fibulin-1.

Fibulin-1 was biotinylated with Sulfo-NHS-LC-Biotin (Pierce Biotechnology) following the manufacturer’s protocol. Biotinylated fibulin-1 was then dialyzed in PBS to remove unbound biotin. Soluble biotinylated fibulin-1 was incubated with 2× molar ratio of ADAMTS-1, catalytically inactive ADAMTS-1 (zinc mutant), or buffer control in 50 mM Tris, pH 7.4, 10 mM CaCl₂, 80 mM NaCl overnight at 37 °C in a total volume of 60 μl. Biotinylated fibulin-1 was also incubated in fibronectin-coated 96-well plates. Fibronectin-immobilized fibulin-1 was subsequently incubated with 2 molar ratio of ADAMTS-1 or zinc mutant in 50 mM Tris, pH 7.4, 10 mM CaCl₂, 80 mM NaCl overnight at 37 °C in a total volume of 60 μl. Digests were separated on SDSA containing 10% polyacrylamide gels under reducing conditions with β-mercaptoethanol. Biotinylated proteins were detected with avidin-conjugated horseradish peroxidase (Vector Labs).

Proteoglycan Digestion—Rat aggrecan was a generous gift from Dr. John Sandy (Shriners Hospital for Children, Tampa, FL). Aggrecan is a proteoglycan secreted from intervertebral disc and cartilage and contains a large number of cartilage proteoglycan core proteins, including aggrecan, decorin, biglycan, and fibromodulin. Aggrecan is a large proteoglycan with a high molecular weight of 250–300 kDa, which is composed of a glycosaminoglycan (GAG) and a core protein. The core protein contains a highly glycosylated N-terminal domain, a central collagen-like domain, and a C-terminal domain. The GAG is a complex polysaccharide that is covalently bonded to the core protein via a covalent linkage at the C-terminal domain.

PCR Genotyping of ADAMTS-1 Mice—Genomic DNA was isolated from tails of embryos using a standard phenol/chloroform extraction protocol. Wild-type locus was amplified using primers that anneal to the 5’-end of exon 9 as mentioned and the 3’-end of exon 7 to the 3’-UTR of the ADAMTS-1 gene. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

Identification of Fibulin-1D by Yeast Two-hybrid Screening as an ADAMTS-1-binding Protein—A yeast two-hybrid screen of a human placenta cDNA library was conducted to identify proteins capable of interacting with ADAMTS-1. A fragment of the carboxyl-terminal portion of human ADAMTS-1 containing the two last thrombospondin type I repeats (amino acid residues 827–951) was used as a bait to screen for potential binding proteins (Fig. 1). Among several positive clones identified, one clone, designated F3.3.2, contained a 1371-bp cDNA fragment for ADAMTS-1 and a 360-bp fragment encoding the 3′-end of fibulin-1.

Generation of ADAMTS-1 Null Mice—Bacterial artificial chromosome (BAC) clone number 413009 carrying genomic fragments of adams1 from mouse strain 129/SvJ was purchased from Genome Systems, Inc. (St. Louis, MI). An 8.4-kb EcoRI fragment extending from the 5′-untranslated region (UTR) to exon 2 and a 6.6-kb HindIII fragment spanning from intron 7 to the 3′-UTR were obtained after digestion of BAC clone number 413009 and were subsequently subcloned into pGEM11ZF(+) and pGEM7Zf(+). Promega, Madison, WI, respectively. The targeting construct was generated by subcloning a 3.2-kb HindIII-Xbal fragment spanning the 5′-UTR to part of intron 1, a 1.8-kb BamHI-BamHI fragment encompassing exon 9 and the 3′-UTR and a 2.0-kb floc-PGK-Neo (Xbal) into pGEM11Zf(+). The floc-PGK-Neo construct was a generous gift from Dr. K. Lyons. The resulting targeting construct was linearized with Xhol and HindIII to remove the pGEM11Zf(+)-plasmid prior to being introduced into 129/SvJ-derived embryonic stem cells by electroporation at the UCLA Molecular Genetics and Technology Center. Embryonic stem cells were then selected in growth medium containing G418 (300 μg/ml) and homologous recombinaats were identified by Southern blot analysis. Chimeric males were generated from two independent targeted embryonic stem cell clones injected into C57BL/6 blastocysts (UC Irvine Transgenic Mouse Facility, Irvine, CA). Male chimeras were crossed with BALB/c females, and germ line transmission was obtained from both independent embryonic stem clones.

PCR Genotyping of ADAMTS-1 Mice—Genomic DNA was isolated from tails of embryos using a standard phenol/chloroform extraction protocol. Wild-type locus was amplified using primers that anneal to the 5′-end (5′-TGCCACACCTCACCTGCTTAC-3′) and the 3′-end of exon 9 (5′-TTAATGACACCTCGTCTCC-3′). Targeted locus was amplified using primers to the 3′-end of exon 9 as mentioned and the 5′-PGK-neo cassette sequence (5′-TATCGGCCCCTCCGAGATT-3′). PCR was performed using HOTMASTER Taq polymerase (Eppendorf AG, Hamburg, Germany) with annealing temperature of 62 °C for 35 cycles.

RESULTS

Identification of Fibulin-1D by Yeast Two-hybrid Screening as an ADAMTS-1-binding Protein—A yeast two-hybrid screen of a human placenta cDNA library was conducted to identify proteins capable of interacting with ADAMTS-1. A fragment of the carboxyl-terminal portion of human ADAMTS-1 containing the two last thrombospondin type I repeats (amino acid residues 827–951) was used as a bait to screen for potential binding proteins (Fig. 1). Among several positive clones identified, one clone, designated F3.3.2, contained a 1371-bp insert that
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FIGURE 2. Demonstration of ADAMTS-1-fibulin-1 binding. A, immunoblot of ADAMTS-1 bound to Dynal magnetic beads incubated with fibulin-1 (fib-1) or BSA. B, immunoblot of ADAMTS-1 (87-kDa) using GPB pulled down with fibulin-1C immunoprecipitated from conditioned medium of cells expressing fibulin-1C. Immunoblot of fibulin-1C (fib-1C) was done with mAb 3A11. C, enzyme-linked immunosorbent assays in which the first series shows the binding to soluble 87-kDa ADAMTS-1 to immobilized fibulin-1 or BSA, and the second series shows the binding of soluble fibulin-1 to immobilized 87-kDa ADAMTS-1 or BSA. IMM, immobilized; Sol, soluble. D, enzyme-linked immunosorbent assay showing binding of a range of fibulin-1 concentrations to immobilized ADAMTS-1, fibronectin (FN), or BSA.

FIGURE 3. Fibulin-1 interaction detected by ADAMTS-1 affinity chromatography and co-immunoprecipitation analysis. A, anti-fibulin-1 immunoblot of fractions eluted from ADAMTS-1-Sepharose column after application of placental extract. B, immunoblots showing that ADAMTS-1 co-immunoprecipitates with fibulin-1 and conversely that fibulin-1 co-immunoprecipitates with ADAMTS-1. EXP, exposure.

corresponded to nucleotides 1439–2810 of a human fibulin-1D cDNA (GenBank™ accession number AF126110). The sequence encoded by clone F3.3.2 corresponds to the carboxyl-end of fibulin-1D (amino acid residues 476–703), including the two last epidermal growth factor-like elements and the carboxy-terminal fibulin-type module (Fig. 1).

Evaluation of the ADAMTS-1-Fibulin-1 Binding Interaction.—The interaction between ADAMTS-1 and fibulin-1 was further validated by several biochemical approaches. Tosyl-activated magnetic beads coupled to fibulin-1 were able to bind to purified ADAMTS-1 in solution, whereas BSA-coated magnetic beads did not (Fig. 2A). Fibulin-1C immunoprecipitated from conditioned medium with mAb 3A11 was able to pull down ADAMTS-1 (Fig. 2B, lane 2). Anti-fibulin-1 mAb 3A11 only recovered trace amounts of ADAMTS-1 when in the absence of fibulin-1C (Fig. 2B, lane 5). In addition, the interaction was tested by enzyme-linked immunosorbent assay using purified proteins. As shown in Fig. 2C, fibulin-1 bound to immobilized ADAMTS-1. The reciprocal experiment was also performed, and ADAMTS-1 was found to bind to immobilized fibulin-1 (Fig. 2C). The binding affinity between these two proteins was also determined. Fibronectin was used as a positive control because it has been shown to interact with fibulin-1 (19, 26). 96-well plates were coated with human fibronectin, ADAMTS-1, or BSA (negative control) at 3 μg/ml and subsequently allowed to bind fibulin-1 at varying concentrations. Based on a fit of the data to a form of the binding isotherm (26), fibulin-1 bound to ADAMTS-1 with an apparent dissociation constant \( K_D \) of 1.0 μM as compared with \( K_D \) of 0.43 μM for fibulin-1 binding to fibronectin (Fig. 2D). As previously shown for fibulin-1-fibronectin interactions (19), saturable binding was not achieved between fibulin-1 and immobilized ADAMTS-1. The basis for this may relate to the fact that fibulin-1 can self associate (19); thus, with increased binding of solution phase fibulin-1 to the immobilized ADAMTS-1, additional fibulin-1 binding sites are created. The interaction between fibulin-1 and ADAMTS-1 was also tested using ADAMTS-1-Sepharose affinity chromatography of placental extracts. As shown in Fig. 3A, low ionic strength buffer released weakly interacting fibulin-1 (fractions 3–13). Subsequent exposure to higher pH and high ionic strength buffer released tightly bound fibulin-1 (fraction 22). No fibulin-1 binding was observed when the plain Sepharose column used to preclear the extract was exposed to the same elution protocol.

Finally, we performed co-immunoprecipitation analyses using placental extracts and fibulin-1 monoclonal antibodies. These experiments revealed that ADAMTS-1 could be co-immunoprecipitated with fibulin-1 (Fig. 3B, lane 3). Conversely, fibulin-1 was found to co-immunoprecipitate with ADAMTS-1 antibodies (Fig. 3B, lane 1, GPB). ADAMTS-1 or fibulin-1 were not present in immunoprecipitations using control mouse IgG (lane 2). Together these findings support the conclusion that fibulin-1 binds to ADAMTS-1. However, the biological significance of this interaction is limited to situations where both these proteins are localized. Thus the next step required a careful analysis of expression and localization.

ADAMTS-1 Colocalizes with Fibulin-1 in Vivo.—The relationship between fibulin-1 and ADAMTS-1 expression was first evaluated by Northern analysis. Interestingly, both transcripts displayed a similar
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FIGURE 4. Expression patterns and colocalization of ADAMTS-1 and fibulin-1. A, Northern blot analysis for ADAMTS-1. Poly(A+)RNA was isolated from E16.5–18.5 mouse organs, transferred to nylon membranes, and probed with ADAMTS1 cDNA. Lane 1, kidney; lane 2, lung; lane 3, liver; lane 4, heart; lane 5, skin; lane 6, brain and membranous bone. Poly(A+)RNA from the same samples was also probed in separate membrane with a fibulin-1 probe. Lane 1, kidney; lane 2, lung; lane 3, liver; lane 4, spleen; lane 5, heart; lane 6, skin; lane 7, brain and membranous bone. Both membranes were also probed with a housekeeping cDNA (36B4) to assess loading and transfer efficiency. B, characterization of GP12, a novel antibody for ADAMTS-1. Western blot analysis with (+) purified ADAMTS-1; lane 1, lysates from T47D control cells; lane 2, lysates from T47D cells expressing ADAMTS-1; lane 3, conditioned medium from T47D control cells; lane 4, conditioned medium from T47D cells expressing ADAMTS-1. Images on the right are immunohistochemistry with GP12 antibody on kidney sections from E15.5 wild-type and ADAMTS-1 null mice littersmates. *, nonspecific band. C, fluorescence immunolocalization of fibulin and ADAMTS-1 in the developing kidney. Sections were co-incubated with anti-fibulin-1 (Rb 1323) and anti-ADAMTS-1 (GP12) antibodies. Detection was performed with fluorescein isothiocyanate and CY3-conjugated antibodies, respectively. Sites of colocalization are indicated by the arrows and can be visualized in yellow in the merged image on the right column. Panels a–c, low magnification (×100); d–l, high magnification (×400).

FIGURE 5. Fibulin-1 is not a substrate for ADAMTS-1. A and B, fibulin-1 was incubated with ADAMTS-1 at 1:1, 1:2, and 1:3 molar ratios and aliquots of reaction mixtures immunoblotted using antibodies recognizing the amino-terminal region of fibulin-1 (mAb 3A11) (A) and antibodies to the carboxyl-terminal region of fibulin-1C (mAb 5D12) (B). C and D, biotinylated fibulin-1 was evaluated as ADAMTS-1 substrate. Both soluble biotinylated fibulin-1C and biotinylated fibulin-1 immobilized to fibronectin (FN) were incubated with 2-fold molar excess of ADAMTS-1, catalytically inactive ADAMTS-1 (Zinc mt), or with buffer only. Detection of biotinylated fibulin-1 was done with avidin-conjugated horseradish peroxidase. FN, fibronectin.

expression profile, at least in the organs evaluated from mouse embryos (Fig. 4A). Because the highest level of expression for both transcripts was noted in the kidney, we pursued immunohistochemical analysis in this organ using anti-fibulin-1 antibodies (24) and a newly developed ADAMTS-1 antibody (Fig. 4B). At day E15.5 of embryonic development, fibulin-1 localized to basement membrane of the developing nephron (Fig. 4C, panels d and g, arrows). This expression pattern correlated with the distribution of ADAMTS-1 protein, which was also detected in discrete patches within the basement membrane at this stage of development (panels e and h, arrows). It is important to emphasize that this colocalization was indeed patchy; in fact, ADAMTS-1 is not expressed in the adult kidney at that site.4 The findings indicate that ADAMTS-1 is present in a relatively narrow window of time during the morphogenesis of the epithelial tubules in the developing kidney. This is consistent with previous findings by in situ hybridization (25).

Fibulin-1 Is Not Cleaved by ADAMTS-1—We next tested whether fibulin-1 was a substrate for ADAMTS-1. Immunoblot analysis using two fibulin-1 antibodies showed no cleavage product nor was the amount of full-length fibulin-1 diminished by incubation with ADAMTS-1 (Fig. 5, A and B). To address the possibility that digestion destroyed fibulin-1 antibody reactive epitopes, we incubated biotinylated fibulin-1 with ADAMTS-1 and detected the digests using avidin-conjugated HRP (Vector Labs). No cleavage products were detected using this approach (Fig. 5C). Because fibulin-1 is an ECM protein, we speculated that conformational changes associated with ECM interaction could expose cleavage sites. We therefore allowed fibulin-1 to bind

4 S. N.-M. Thai and M. L. Iruela-Arispe, unpublished observations.
to immobilized fibronectin and evaluated the ability of ADAMTS-1 to mediate fibulin-1 cleavage. As shown in Fig. 5D, fibronectin-immobilized fibulin-1 was also not cleaved by ADAMTS-1. Taken together, the findings indicate that fibulin-1 is not a substrate of ADAMTS-1.

Fibulin-1 Enhances the Catalytic Activity of ADAMTS-1—Because fibulin-1 was not cleaved by ADAMTS-1, we speculated that the interaction might modulate the function of one or another. Fibulin-1 has been shown to bind the C-type lectin domains of aggrecan and versican (20, 22). These two proteoglycans are recognized substrates for ADAMTS-1 (9–11). Thus, we investigated the possibility that fibulin-1 might modulate the proteolytic activity of ADAMTS-1 toward aggrecan. In the presence of fibulin-1, full-length aggrecan was completely cleaved to the 250-kDa fragment by ADAMTS-1 (Fig. 6A). In contrast, inclusion of other proteins (fibronectin, vitronectin, laminin-1, and BSA) resulted in incomplete digestion of the full-length aggrecan over the same time period (Fig. 6A). Fibronectin was also able to enhance ADAMTS-1 catalytic activity (~2-fold over BSA); however, it was not as efficient as fibulin-1. The other ECM proteins tested failed to enhance ADAMTS-1 proteolytic activity. None of the ECM proteins tested (i.e. fibulin, laminin-1, fibronectin, and vitronectin) leads to an inhibition of ADAMTS-1 aggrecanase activity (Fig. 6A).

Next, we evaluated the effect of fibulin-1 on the kinetics of aggrecan proteolysis. In this figure, the degree of contamination with the 250-kDa form was ~50% (see “Experimental Procedures”), yet it is clear that addition of ADAMTS-1 processed the intact aggrecan to completion, as early as 30 min when in the presence of fibulin-1. In addition, the lower 65-kDa aggrecan species was also detected at 30 min, and levels increased progressively with time. By contrast, digestion was still incomplete after 120 min when laminin-1 was present (Fig. 6B). At the same time point, aggrecan cleavage product (65 kDa) with fibulin-1 was 4-fold greater than with laminin-1 (Fig. 6B).

The effect of fibulin-1 protein concentration on the cleavage reaction was also tested. These studies showed that the extent of aggrecan cleavage to release the 250- and 65-kDa fragments was proportional to the concentration of fibulin-1 in the reaction (Fig. 6C). The enhancement of ADAMTS-1 catalytic activity was specific to fibulin-1, as increasing concentrations of laminin-1 failed to augment ADAMTS-1 activity (Fig. 6C). Furthermore, the use of gradient gels revealed that presence of fibulin-1 significantly altered the mobility of aggrecan (Fig. 6C, compare lanes 1 and 2, brackets). Thus, from a high molecular mass complex in the absence of fibulin-1, aggrecan resolves into a dual lower molecular mass doublet. This change was dependent on levels of fibulin-1 and did not occur with other ECM proteins.

FIGURE 6. Proteolytic cleavage of aggrecan by ADAMTS-1 is enhanced by fibulin-1. A, anti-G1 (Globular domain 1 of aggrecan) immunoblot of digest reactions of aggrecan incubated with the various ECM proteins in the presence or absence of ADAMTS-1. The digests were resolved by 10% SDS-PAGE. B, anti-G1 immunoblot of digest reactions of aggrecan incubated with ADAMTS-1 in the presence of fibulin-1 or laminin for the indicated time periods. The digests were resolved by 10% SDS-PAGE. Arrow in panels A and B indicate full-length aggrecan. Arrowheads indicate proteolytic species of 250 and 65 kDa. C, anti-G1 immunoblot of digest reactions of aggrecan incubated with ADAMTS-1 in the presence of increasing amounts of fibulin-1 or laminin. The digests were resolved by 4–12% gradient SDS-PAGE. D, anti-G1 immunoblot of digest reactions of aggrecan with increasing levels of fibulin-1 in the presence or absence or ADAMTS-1. The digests were resolved by 4–12% gradient SDS-PAGE. Arrowheads in panels C and D indicate the high molecular mass intact aggrecan. Lower arrow and arrowhead within the bracket show the shift in mobility of aggrecan when in the presence of fibulin. ADAMTS-1 mediates the cleavage of those forms to a tight 250-kDa band and a 65-kDa band (indicated by arrowheads).
FIGURE 7. Generation of ADAMTS-1 null mouse and evaluation of aggrecanase activity in the kidney of wild-type and ADAMTS-1 knock-out embryos. A, schematic diagram of gene targeting strategy to disrupt the ADAMTS-1 locus. B, representative Southern blot of embryonic stem clones transformed with ADAMTS-1-flox-PGK-Neo targeting construct showing the correct site of integration. C, representative gel of PCR used to determine genotype of embryos derived from heterozygous ADAMTS-1 crosses. D, fluorescence immunohistochemical analysis of aggrecan (panels a and d, in green), ADAMTS-1 (panels b, e, h, and k, in red), and proteolyzed aggrecan (panels g and j, in green). Evaluation was performed in littermates of E15.5 of wild-type (a–c, g–i) or null genotype (d–f, j–l) as indicated on the left of the panels. Sites of colocalization are indicated by the arrows and can be visualized in yellow in the merged image on the right column. Magnification in all panels, ×400.
not change after the ratio of both proteins reached equality (i.e. 1:1, aggrecan to fibulin). In contrast, the presence of laminin at 1:1 molar ratio did not result in the same mobility shift, although aggrecan appeared as a high molecular mass smear. The data indicate that fibulin-1 alters the conformation of aggrecan in a manner that is not affected by exposure to detergents. Although fibulin-1 significantly affected the mobility of aggrecan, processing of the proteoglycan required ADAMTS-1 (Fig. 6D).

**ADAMTS-1 Is a Biologically Important Aggrecanase in the Developing Kidney**—Our previous results demonstrated colocalization of fibulin and ADAMTS-1 in the developing kidney tubules, and thus we sought to determine whether this enzyme is relevant for the processing of aggrecan at similar sites. To answer this question we inactivated the ADAMTS-1 gene by homologous recombination in mouse (Fig. 7, A–C). Although the targeting vector was slightly different from those described by other investigators (13, 14), the phenotype is very similar to previously described ADAMTS-1 null mice. Absence of ADAMTS-1 results in significant lethality at the intraembryonic/neonatal stage. Mendelian distribution of heterozygous crosses indicates that 40% of homozygous animals for the null mutation are not recovered (assessment done in >100 offspring of mixed background). The remaining homozygous animals display kidney pathologies and urinary track obstructions. In addition, ADAMTS-1 null mice have lower weight mass than littermate wild-type controls (wild-type 28.83 g average from littermate males 4–6 months of age). We evaluated expression of aggrecan and ADAMTS-1 in wild-type and ADAMTS-1 null littersmates at E15.5 (Fig. 7D). A significant degree of colocalization was detected in the apical epithelial tubules as well as in the basement membrane (Fig. 7D, panels a–c), a site where we also found fibulin-1 (Fig. 4C). Expression of aggrecan was not altered in the absence of ADAMTS-1 (Fig. 7D, panels d–f). Interestingly, using a neo-epitope antibody to recognize cleaved aggrecan (11), we were also able to detect colocalization with ADAMTS-1 (panels g–i). However, cleaved aggrecan was poorly detected in the absence of ADAMTS-1 (panels j–l). These results indicate that, at least in the kidney, ADAMTS-1 is a biologically relevant aggrecanase.

**DISCUSSION**

Using a yeast two-hybrid screen, we demonstrated that fibulin-1 binds to ADAMTS-1 through the last three epidermal growth factor-like repeats in fibulin-1 and thrombospondin type I repeats 2 and 3 in ADAMTS-1. Interaction of these proteins was further confirmed by several biochemical assays, including pulldown, solid phase binding, and co-immunoprecipitation, and it was also validated several biochemical assays, including pulldown, solid phase binding, and co-immunoprecipitation, and it was also validated ADAMTS-1. Interaction of these proteins was further confirmed by several biochemical assays, including pulldown, solid phase binding, and co-immunoprecipitation, and it was also validated.

The thrombospondin type I repeats in ADAMTS-1 have been shown to interact with heparin and are also likely to bind to other ECM proteins (35). Removal of this domain increases the solubility of the enzyme in tissue culture conditions and decreases its catalytic activity (36, 37). In contrast, removal of the carboxyl-terminal region in ADAMTS-4 improves the activity of this molecule (38, 39). Thus, it appears that the function of the carboxyl-terminal region might differ in different ADAMTS proteins. Recently, fibronectin was found to bind ADAMTS-4 via the carboxyl-terminal region. More importantly, this binding inhibits ADAMTS-4 proteolytic activity toward aggrecan (40).

Contrary to our expectations, fibulin-1 did not hinder the activity of ADAMTS-1 nor was it found to be a substrate for the enzyme. Unlike the interaction between fibronectin and ADAMTS-4, fibulin-1 significantly enhanced the catalytic activity of ADAMTS-1. Interestingly, we found that fibronectin could also increase ADAMTS-1-mediated proteolytic cleavage of aggrecan but not to the same degree as fibulin-1 (Fig. 6A). Cofactor activity has been observed for other ECM proteins. For example, procollagen C-proteinase enhancer increased the proteolytic processing of procollagen by bone morphogenetic protein-1, an activity that required binding of the cofactor to the substrate (34).

The similar in vivo expression patterns of ADAMTS-1 and fibulin-1 indicate that this interaction could be of physiological relevance. Fibulin-1 and ADAMTS-1 were found colocalized in the kidney basement membrane (Fig. 4C, arrows). These findings are consistent with studies that independently demonstrated expression of transcripts for fibulin-1 and ADAMTS-1 in the developing nephron and several epithelial structures (41, 25). Interestingly, mice that lack fibulin or ADAMTS-1 die from kidney abnormalities (13, 14, 23).

Surprisingly, we did not detect ADAMTS-1 in developing cartilage, a prominent site for fibulin-1 and aggrecan. Thus, the participation of ADAMTS-1 in aggrecan proteolysis appears to be tissue specific. In fact, a recent study convincingly demonstrated that ADAMTS-1 does not participate in aggrecan turnover in the growth plate and articular cartilage, a site where expression of ADAMTS-4 and 5 predominates (42, 43). In the kidney, however, the roles appear reversed, as expression of ADAMTS-4 and 5 is non-detectable. Supporting an aggrecanase activity for ADAMTS-1 in the kidney, we found diminished-to-undetectable levels of aggrecan processing in the ADAMTS-1 null mice in comparison to control (Fig. 7). These findings might bear significance to the kidney phenotype displayed by the ADAMTS-1 null mouse.

ADAMTS-1 is significantly up-regulated by inflammatory mediators, particularly interleukin-1 and lipopolysaccharide (44, 25). In fact, inflammatory vascular pathologies such as atherosclerosis showed high levels of ADAMTS-1 and fibulin-1 (45, 46). Thus, fibulin-1 could play an important role in the degradation of proteoglycans by ADAMTS-1 during pathological conditions induced by inflammatory processes.

A genetic demonstration of the interaction between fibulin-1 and members of the ADAMTS family has been recently shown in Caenorhabditis elegans (47). These authors found that the ADAM protease GON-1, and fibulin-1 have antagonistic roles in the regulation of gonad morphogenesis. Together, our findings and those on GON-1 suggest that fibulin has affinity for and is a relevant partner of multiple ADAMTS proteases.

Several ECM molecules are known to regulate the activity of matrix metalloproteinases (34, 33). Our findings offer the first demonstration of...
ADAMTS-1 Function Is Enhanced by Fibulin-1

an enhancer for the ADAMTS family of proteases that is likely to play a relevant role in the morphogenesis of the kidney epithelium.

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Fibulin-1 Acts as a Cofactor for the Matrix Metalloprotease ADAMTS-1
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