Proteolytic Activities of Human ADAMTS-5
COMPARATIVE STUDIES WITH ADAMTS-4*

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Aggrecanases have been characterized as proteinases that cleave the Glu373-Ala374 bond of the aggrecan core protein, and they are multidomain metalloproteinases belonging to the ADAMTS (adamalysin with thrombospondin type 1 motifs) family. The first aggrecanases discovered were ADAMTS-4 (aggrecanase 1) and ADAMTS-5 (aggrecanase 2). They contain a zinc catalytic domain followed by non-catalytic ancillary domains, including a disintegrin domain, a thrombospondin domain, a cysteine-rich domain, and a spacer domain. In the case of ADAMTS-5, a second thrombospondin domain follows the spacer domain. We previously reported that the non-catalytic domains of ADAMTS-4 influence both its extracellular matrix interaction and proteolytic activities. Here we report the effects of these domains of ADAMTS-5 on the extracellular matrix interaction and proteolytic activities and compare them with those of ADAMTS-4. Although the spacer domain was critical for ADAMTS-4 localization in the matrix, the cysteine-rich domain influenced ADAMTS-5 localization. Similar to previous reports of other ADAMTS family members, very little proteolytic activity was detected with the ADAMTS-5 catalytic domain alone. The sequential inclusion of each carboxy-terminal domain enhanced its activity against aggrecan, carboxymethylated transferrin, fibromodulin, decorin, biglycan, and fibronectin. Both ADAMTS-4 and -5 had a broad optimal activity at pH 7.0–9.5. Aggrecanolytic activities were sensitive to the NaCl concentration, but activities on non-aggrecan substrates, e.g. carboxymethylated transferrin, were not affected. Although ADAMTS-4 and ADAMTS-5 had similar general proteolytic activities, the aggrecanase activity of ADAMTS-5 was at least 1,000-fold greater than that of ADAMTS-4 under physiological conditions. Our studies suggest that ADAMTS-5 is a major aggrecanase in cartilage metabolism and pathology.

Destruction of articular cartilage is a feature of various arthritides, including rheumatoid and osteoarthritis, that results in joint impairment and disability. It is caused primarily by an elevation in proteolytic enzymes that degrade macromolecules of the cartilage extracellular matrix. Aggrecan degradation is initially observed followed by essentially irreversible collagen degradation. The proteinases that are responsible for aggrecan degradation in cartilage are metalloproteinases (MMPs) and “aggrecanases,” members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin type 1 motifs) family (1, 2). Aggrecanase activity was first defined as the ability to cleave the Glu373-Ala374 bond in the interglobular domain (IGD) of the aggrecan core protein (3, 4). The first two proteinases shown to be capable of cleaving aggrecan at this site were ADAMTS-4 (aggrecanase 1) (5), and ADAMTS-5 (aggrecanase 2) (6). More recently, ADAMTS-1, -8, -9, -15, -16, and -18 were shown to cleave the Glu373-Ala374 bond in the IGD at a high enzyme-substrate ratio (2, 7). Although this bond is also cleaved by MMP-8 (8) and MMP-14 (9) at high concentration in vitro, the primary MMP cleavage site in the IGD is considered to be at the Asn341-Phe342 bond (1). The cleavage of the Glu373-Ala374 bond in IL-1-stimulated pig articular cartilage explants is blocked by tissue inhibitor of metalloproteinases 3 (TIMP-3) but not by other TIMPs (10). This is a further implication of ADAMTS metalloproteinases as the proteinases responsible for the observed aggrecanase activity.

Among the ADAMTSs that have aggrecanase activity, ADAMTS-4 has received more attention than other ADAMTSs because the elevation of its mRNA is readily observed in chondrocytes treated with IL-1, whereas ADAMTS-5 mRNA is not (11, 12). However, ADAMTS-4-null mice did not exhibit any significant protective effect on cartilage aggrecan loss compared with the wild-type mice when challenged in an osteoarthritis model induced by surgical joint destabilization (13). Similarly ADAMTS-1-null mice were not protected against joint destruction in an antigen-induced arthritis model (14). These studies suggest that neither

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4 The abbreviations used are: MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin type 1 motifs; anti-TS5cat, rabbit anti-ADAMTS-5 catalytic domain; Cm-Tf, reduced, carboxymethylated transferrin; CysR, cysteine-rich; Dis, disintegrin; DMEM, Dulbecco’s modified Eagle’s medium; IGD, interglobular domain of aggrecan; IL-1, interleukin-1; MT-MMP, membrane-type matrix metalloproteinase; N-TIMP, amino-terminal domain of tissue inhibitor of metalloproteinases; Sp, spacer; TIMP, tissue inhibitor of metalloproteinases; TS, thrombospondin type 1; MES, 4-morpholineethanesulfonic acid; ECM, extracellular matrix.

5 ADAMTS-5 was originally referred to as “ADAMTS-11.”
ADAMTS-4 nor ADAMTS-1 may be the major enzyme that causes aggrecan loss during the progression of arthritis. On the other hand, studies with ADAMTS-5-null mice showed that the lack of ADAMTS-5 protected against cartilage destruction when osteoarthritis (15) or inflammatory arthritis (16) was induced. This suggests that ADAMTS-5 plays a key role in aggrecan degradation at least in these models of arthritis in mice.

ADAMTS-4 and ADAMTS-5 are multidomain metalloproteinases secreted from the cell into the extracellular space. Both enzymes have a similar domain arrangement consisting of a prodomain, a catalytic metalloproteinase domain, a disintegrin (Dis) domain, a thrombospondin type 1 (TS) domain, a cysteine-rich (CysR) domain, and a spacer (Sp) domain. In addition, ADAMTS-5 contains an extra TS domain after the spacer domain (see Fig. 1 for domain arrangements).

We have shown previously that the non-catalytic ancillary domains of ADAMTS-4 play a major role in regulating aggrecanase activity (17). In particular, full-length ADAMTS-4 digests the aggrecan core protein most effectively but has little activity to cleave the initially characterized aggrecanase cleavage site, Glu373-Ala374 (17, 18), or other non-aggrecan protein substrates (17). When the carboxyl-terminal Sp domain is removed, the enzyme gains activity for the Glu373-Ala374 bond as well as new proteolytic activities against proteins such as reduced, carboxymethylated transferrin (Cm-Tf), fibromodulin, and decorin (17). ADAMTS-4 is converted to a molecular size similar to that of our Sp deletion mutant by MMP-17 (MT4-MMP) in chondrocytes (18) and cartilage explants (19). This suggests that the removal of the ADAMTS-4 spacer domain and hence increased ADAMTS-4 proteolytic capabilities occur in vivo. However, little proteolytic activity was detected with the metalloproteinase domain alone (17).

Unlike ADAMTS-4, the biochemical characterization of ADAMTS-5 is limited. The only available studies investigate its ability to degrade aggrecan, brevican, and other non-aggrecan protein substrates (20–23). We therefore investigated the functional significance of the non-catalytic domains of ADAMTS-5 by expressing recombinant enzymes where we have systematically deleted the ancillary domains from the carboxyl terminus. We then measured their enzymatic activities for the aggrecan core protein, Cm-Tf, and other cartilage matrix macromolecules. These studies indicated that ADAMTS-5 is able to cleave aggrecan core protein about 1,000 times more effectively than ADAMTS-4. Furthermore this work revealed that the roles of the non-catalytic domains for aggrecanolytic activities and the salt sensitivity between the two enzymes are significantly different. Our results provide biochemical evidence to support that ADAMTS-5 is the major aggrecanolytic metalloproteinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human embryonic kidney cells (HEK 293-EBNA), pCEP4 vector, and the Zero Blunt TOPO PCR kit were from Invitrogen. G418 and hygromycin B were from PAA Laboratories (Somerset, UK). Macro-Prep 25 S resin and pre-stained and unstained Precision Protein Standards for SDS-PAGE were from Bio-Rad. Anti-rabbit alkaline phosphatase-linked antibody, anti-mouse alkaline phosphatase-linked antibody, and alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl 1-phosphate and nitroblue tetrazolium) were from Promega (Southampton, UK). Decorin, biglycan, fibromodulin, anti-FLAG M2-agarose, FLAG peptide, and human transferrin were from Sigma-Aldrich. Restriction enzymes were from New England Biolabs (Hitchin, UK). Pfu DNA polymerase, GEX Gel Band PCR Purification kit, and GEX Microplasmid Purification kit were from Amersham Biosciences. pCR-Script and pSG5 were from Stratagene (Amsterdam, The Netherlands). FuGENE 6 was from Roche Applied Science. Recombinant human N-TIMP-1 (the amino-terminal domain of tissue inhibitor of metalloproteinase-1), human TIMP-2, and human N-TIMP-3 were prepared as described previously (see Ref. 10). Aggrecan was purified from bovine nasal cartilage according to the method of Hascall and Sajdera (24). Gelatin was prepared by heat denaturing guinea pig type I collagen (25). Human fibronectin was purified from expired human plasma as described previously (26). Monoclonal antibodies BC-3 that recognizes the newly generated amino-terminal 374-ARGSV epitope of the aggrecan core protein and 2-B-6 that recognizes the chondroitinase-resistant chondroitin 4-sulfate stubs of aggrecan core protein were generated as described previously (27, 28). The rabbit anti-GELE antibody that recognizes the new GELE1480 carboxyl terminus generated by aggrecanase cleavage of the aggrecan core protein was raised in a rabbit against the aggrecan peptide sequence CGGTAGELE (amino acids 1475–1480) (the amino acids in italics are not part of the aggrecan sequence but added for the purpose of linking the peptide to keyhole limpet hemocyanin). The rabbit anti-ADAMTS-5 catalytic domain (anti-TS5cat) antibody was raised in a rabbit against the peptide sequence CEETFGST-EDKRL (amino acids 410–422) and linked to keyhole limpet hemocyanin. Both peptides were kindly provided by Prof. G. B. Fields (Florida Atlantic University).

**Construction of cDNA Coding for Human ADAMTS-5 and the Carboxyl-terminal Domain Deletion Mutants**—Full-length ADAMTS-5 (TS5-1) was amplified from the cDNA of primary human chondrocytes and subcloned into pCR-Script. Carboxyl-terminal FLAG-tagged full-length ADAMTS-5 (ADAMTS5-1) and its domain deletion mutants were created using the PCR method. The PCR was performed with pCR-Script-ADAMTS-5 as a template and amplified by Pfu Turbo DNA polymerase using two primers: forward primer, 5′-ACTGGTGACACAACTGTCGCTCGGGGTGGG-3′ (ADAMTS5 FW) containing a KpnI restriction site (underlined) and a Kozak consensus sequence (italic); reverse primer, 5′-AGGAGATCTCATGCTATTTATCTCACTCTTTTAATATACATTTCTCAACATATTTCCACATTG-3′ (ADAMTS5-1 RV FLAG), 5′-AGGAGATCTCATGCTATTTATCTCAGTTTTTAAATCAGTTGTTGTTACATTTCTCAGTTG-3′ (ADAMTS5-2 RV FLAG), 5′-AGGAGATCTCATGCTATTTATCATCATC-3′ (ADAMTS5-3 RV FLAG), 5′-AGGAGATCTCATGCTATTTATCTCATCATC-3′ (ADAMTS5-4 RV FLAG), 5′-AGGAGATCTCATGCTATTTATCTCATCATC-3′ (ADAMTS5-5 RV FLAG), 5′-AGGAGATCTCATGCTATTTATCTCATCATC-3′ (ADAMTS5-6 RV FLAG).
Proteolytic Activities of ADAMTS-5

CCAGGAT-3’ (ADAMTS5-6 RV FLAG) containing a BglII restriction enzyme site (underlined), a stop codon, and the FLAG epitope (coding Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys in italics). The PCR was carried out for 35 cycles of denaturation (60 s at 94 °C), annealing (60 s at 55 °C), and extension (3 min 30 s at 72 °C). The PCR products were ligated into the pCR-BLUNT II-TOPO vector using the Zero Blunt TOPO PCR Cloning kit according to the manufacturer’s instructions, sequenced, and then subcloned into both the pCEP4 and pSG5 vectors using the KpnI and BglII restriction enzyme sites that were introduced into the ADAMTS-5 DNA products at the PCR step. A diagram of the ADAMTS-5 constructs (TS5-1 to TS5-6) created is shown in Fig. 1.

Expression and Purification of the Recombinant Human ADAMTS-5 and Its Domain Deletion Mutants—The pCEP4 vector containing full-length human ADAMTS-5 (TS5-1) or its domain deletion mutants was transfected into HEK 293-EBNA cells with FuGENE 6. The stably transfected cells were selected for hygromycin B resistance (100 µg/ml) in DMEM, 10% fetal calf serum, 250 µg/ml G418, 100 units/ml penicillin, and 100 units/ml streptomycin over a period of 3 weeks. To obtain the recombinant protein, the culture medium was replaced with serum-free DMEM containing 0.2% lactalbumin hydrolysate, penicillin, and streptomycin. In the case of TS5-1-, -2-, and -3-producing cells, 100 µg/ml heparin was added into the culture to obtain mature enzyme in the conditioned media. After 3 days, the conditioned media were harvested and stored at −20 °C until purification. A stable HEK 293-EBNA cell line was not successfully established for TS5-6; therefore this enzyme was purified from transiently transfected HTB-94 cells.

The collected conditioned media (1 liter) were filtered to remove any cell debris and passed over a 3-ml anti-FLAG M2-agarose column at 4 °C. The column was first washed extensively with 50 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.02% Brij-35. The bound material was eluted with 200 µg/ml FLAG peptide in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.02% Brij-35. FLAG-eluted TS5-2, TS5-3, TS5-4, TS5-5, and TS5-6 were further purified on a Sephacryl S-200 gel filtration column (1.6 x 100 cm) to remove the FLAG peptide. Due to the low abundance of TS5-1 in the starting material (~30 µg/liter of conditioned media), a Macro-Prep 25 S resin

FIGURE 1. Schematic representation of the ADAMTS-4, -5, and their domain deletion mutants used in this study. The theoretical molecular masses are based on their amino acid composition, whereas the observed molecular masses of the active forms are based on SDS-PAGE analysis. Each proteinase contains a FLAG sequence epitope (DYKDDDDK) at its carboxyl terminus. The arrow indicates the position of the potential furin cleavage site. Pro, prodomain.
was used to remove the FLAG peptide and to concentrate the protein. FLAG-eluted fractions were dialyzed against 20 mM Tris acetate (pH 7.5) containing 250 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.02% Brij-35. ADAMTS5-1 was eluted from the column with 50 mM Tris-HCl (pH 7.5) containing 1 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.02% Brij-35. The TS5-1-containing fractions, monitored using SDS-PAGE, were pooled; dialyzed against aggrecanase reaction buffer (50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.02% Brij-35); snap frozen; and stored at −80 °C.

**Active Site Titration of ADAMTS-5 and Its Mutant**—The concentrations of active ADAMTS-5 and its domain deletion mutants were determined by titration with N-TIMP-3. The concentration of TS4-5 and TS5-6 (both are the catalytic domain only) was determined with Coomassie Brilliant Blue R-250 using bovine serum albumin as standard due to low enzymatic activity.

**Transient Transfection of the Human Chondrosarcoma HTB-94 Cells**—Human chondrosarcoma cells (HTB-94) were seeded at 5 × 10⁵ cells/well of a 12-well plate in media containing 5% fetal calf serum. The cells were grown overnight and transfected the following day with 0.5 μg of DNA using FuGENE 6 according to the manufacturer’s instructions. The next day, the conditioned media were removed, the cells were washed once with serum-free DMEM to remove any remaining serum, and 500 μl of serum-free DMEM with or without 100 μg/ml heparin was added to each culture and incubated for 48 h (the total transfection time was 72 h). The conditioned media were then harvested, and detached cells were removed by centrifugation at 3,000 × g for 10 min. Proteins in the conditioned media were precipitated using a final concentration of 3.3% trichloroacetic acid and subjected to Western blot analysis with the anti-FLAG M2 antibody to detect ADAMTS-5 and its mutants.

**Immunolocalization of ADAMTS-5 in Human Chondrosarcoma HTB-94 Cells**—The procedures were essentially the same as those described for ADAMTS-4 studies (17). HTB-94 cells were transiently transfected with the pCEP4 expression vector containing cDNA encoding ADAMTS-5 or its variants and cultured in serum-free DMEM with or without heparin (100 μg/ml) for 48 h. To localize each recombinant ADAMTS-5 protein, the cells were washed, fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline for 7 min, washed again, and then incubated with blocking solution. The samples were then incubated with the mouse anti-FLAG M2 antibody followed by Alexa-488-conjugated goat anti-(mouse IgG) IgG. The specimens were then washed with phosphate-buffered saline, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline, and incubated with Alexa-660-conjugated phalloidin to visualize actin filaments within the cells. The samples were viewed using a Nikon Eclipse TE 2000-U microscope equipped with a PerkinElmer Life Sciences Ultraview Live Cell Imaging System with excitation and emission wavelengths at 488 and 525 nm for Alexa-488 and at 647 and 700 nm for Alexa-660, respectively.

**RESULTS**

**Expression of the Recombinant ADAMTS-5 and Its Domain Deletion Mutants**

Full-length ADAMTS-5 and its carboxyl-terminal domain-deleted mutants were purified from the conditioned media of stably transfected HEK 293-EBNA or transiently transfected HTB-94 cells by anti-FLAG M2 affinity chromatography. The

**SDS-PAGE and Western Blot Analysis**—SDS-PAGE was run under reducing conditions using a modification of the Ammodi/glycine/HCl buffer system of Wyckoff et al. (29). Proteins were stained either with Coomassie Brilliant Blue R-250 or with silver (30). For Western blot analysis after SDS-PAGE, proteins were electrophoresed onto a polyvinylidene difluoride membrane, and the membrane was processed as described previously (17).

**Aggrecanase Assays**—Aggrecan (750 nm) was incubated with ADAMTS4-2 (TS4-2), full-length ADAMTS-5, or an ADAMTS-5 domain deletion mutant in 100 μl of aggrecanase reaction buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.02% Brij 35) at 37 °C for the indicated period of time. The reactions were stopped by adding an equal volume of double strength glycansaminoglycan buffer (200 mM sodium acetate, 50 mM Tris-HCl (pH 6.8), and 100 mM EDTA). Aggrecan was then deglycosylated by incubating with 0.01 units of chondroitinase ABC/10 μg of aggrecan and 0.01 units of keratanase/10 μg of aggrecan for 16–18 h at 37 °C. The samples were precipitated by adding 5 volumes of acetone, incubated at −20 °C for 15 min, and then centrifuged at 3,000 × g for 10 min. The pellet was dried and dissolved in 20 μl of reducing sample buffer. The products were analyzed by Western blot analysis with the 2-B-6, BC-3, or anti-GELE antibody as described previously (17). For the comparative studies, all polyvinylidene difluoride membranes were processed simultaneously, and Western analyses were carried out under identical conditions. Relative analyses among different forms of ADAMTS-5 and TS4-2 were estimated by visual inspection of Western blots for equivalent amount of products formed based on the concentration of the enzyme and the time of incubation. Gels and blots were scanned using a Bio-Rad GS-710 scanning densitometer, and the band intensity was quantified using the 1D Phoretix quantification software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

**Amino-terminal Sequence Analysis of Cm-Tf and Fibromodulin Fragments**—Cm-Tf (10 mg/ml) was incubated with TS4-2 or TS5-4 in aggrecanase reaction buffer at 37 °C for 72 h, and the products were analyzed by SDS-PAGE. The fragments were electrotransferred to a polyvinylidene difluoride membrane and visualized with Coomassie Brilliant Blue R-250. The bands were excised and placed directly onto a Polybrenetreated glass filter, and samples were analyzed by automated Edman degradation in an Applied Biosystems Procise 494HT sequencer with on-line phenylthiohydantoin high pressure liquid chromatography analysis. Fibromodulin (0.1 mg/ml) was incubated with TS5-4 for 4 h, and the products were deglycosylated with peptide N-glycosidase F prior to SDS-PAGE. The 29-kDa fibromodulin fragment was sequenced in a manner similar to that described above for the Cm-Tf fragments.
FLAG peptide used to elute the bound material was removed either by Macro-Prep 25 S or gel filtration chromatography. All of the preparations exhibited a single band by SDS-PAGE analysis with silver staining except TS5-5, which appeared as two bands due to N-linked oligosaccharides (Fig. 2; see below). The purified proteins were verified to be ADAMTS-5 by Western blot analysis using anti-TS5cat antibody (Fig. 2C).

The molecular masses of the recombinant enzymes estimated by SDS-PAGE were slightly higher than that predicted from their amino acid composition (see Fig. 1), consistent with glycosylation of the protein (7). ADAMTS-5 contains four potential N-glycosylation sites and one potential mucin-type O-glycosylation site. The four N-glycosylation sites were predicted using NetNGlyc 1.0 in the following domains: one in the Dis domain, one in the CysR domain, and two in the Sp domain. One O-glycosylation site was predicted using NetOGlyc 3.1 in the Sp domain. However, these predictions do not explain why the catalytic domain alone (TS5-6) runs at a higher molecular weight than was predicted. TS5-5 always purified as two distinct bands that were both recognized by the anti-FLAG M2 and anti-TS5cat antibodies. The treatment of this sample with peptide N-glycosidase F resulted in a single band of ~39 kDa, indicating that the 41-kDa band is an N-glycosylated form of TS5-5 (Fig. 2D).

The CysR Domain Is Critical for ADAMTS-5 Binding to the Cell Surface and ECM

Immunolocalization of TS5-1 in HTB-94 cells with the anti-FLAG antibody indicated that full-length TS5-1 was associated with the cell surface and ECM. The addition of heparin to the culture medium released the majority of TS5-1 (Fig. 3A). This suggests that TS5-1 binds to negatively charged glycosaminoglycans on the cell surface and in the ECM. Both the carboxyl-terminal TS domain deletion mutant (TS5-2) and the Sp and TS domain deletion mutant (TS5-3) were also localized to the ECM, but further deletion of the CysR domain (TS5-4) released the enzyme into the medium without heparin (Fig. 3B). These data suggest that the CysR domain of ADAMTS-5 plays a significant role in ECM binding.

Proteolytic Activities of ADAMTS-5 and Its Domain Deletion Mutants: Comparative Studies with ADAMTS-4

All studies were conducted with equal concentrations of ADAMTS-4 and ADAMTS-5 as determined by active site titra-
tion with N-TIMP-3. The only exception is TS5-6, whose proteolytic activity was negligible. Therefore, the concentration of TS5-6 was determined by Coomassie Brilliant Blue R-250 staining with bovine serum albumin as standard.

ADAMTS4-2 (TS4-2), which lacks the carboxyl-terminal Sp domain, was used for the comparison with ADAMTS-5 because this form of

FIGURE 4. Detection of the aggrecanase activities of ADAMTS-4 and -5 using aggrecan neoepitope antibodies. Bovine nasal aggrecan (750 nM) was incubated with each recombinant enzyme for the indicated periods of time at 37 °C. The reactions were terminated with 50 mM EDTA, and the samples were deglycosylated and then subjected to SDS-PAGE (6% total acrylamide) followed by Western blot analysis using the following antibodies: the 2-B-6 antibody, which recognizes the chondroitinase-generated chondroitin 4-sulfate stubs remaining on the aggrecan molecule after deglycosylation (A); the BC-3 antibody, which recognizes aggrecanase-generated aggrecan fragments beginning with 374ARGS at the amino terminus (B); and the GELE antibody, which recognizes aggrecanase-generated aggrecan fragments ending with GELE1480 at the carboxyl terminus (C). I–IV, fragments generated by both ADAMTS-4 and ADAMTS-5; Ia, fragment specific to ADAMTS-4.

TABLE 1
Relative proteolytic activities of ADAMTS-4 and ADAMTS-5

| Enzyme form | Aggrecan digestion | Cm-Tf activity |
|-------------|---------------------|----------------|
|             | General activity    | Glu373-Ala374 | Glu1480-Ala1481 |  |
| TS5-1 (Cat-Dis-TS-CysR-5p-TS) | 125,000 | 60,000 | 20,000 | 200 |
| TS5-2 (Cat-Dis-TS-CysR-5p) | 150,000 | 30,000 | 20,000 | 200 |
| TS5-3 (Cat-Dis-TS-CysR-R) | 34,000 | 15,000 | 200 | 200 |
| TS5-4 (Cat-Dis-TS) | 340 | 300 | <10 | 50 |
| TS5-5 (Cat-Dis) | <30 | ND | ND | 25 |
| TS6-6 (Cat) | ND | ND | ND | ND |
| TS4-1 (Cat-Dis-TS-CysR-R) | 125 | <5 | 100 | 100 |
| TS4-2 (Cat-Dis-TS-CysR-R) | 100 | 100 | 100 | 100 |
| TS4-3 (Cat-Dis-TS) | 40 | 60 | 70 | 100 |
| TS4-4 (Cat-Dis) | 3 | ND | ND | 30 |
| TS4-5 (Cat) | ND | ND | ND | ND |
ADAMTS-4 has the highest level of general proteinase activity as compared with full-length ADAMTS-4. Furthermore TS4-2 retains about 80% of the general aggrecanase activity while exhibiting the highest activity against the Glu373-Ala374 bond of the aggrecan IGD.

**Aggrecan Digestion**—Aggrecanolytic activities of the various forms of ADAMTS-5 were tested by detecting digestion products using the following antibodies: (i) the 2-B-6 antibody that recognizes the chondroitinase-resistant chondroitin 4-sulfate disaccharide stubs of the aggrecan core protein (a measure of general aggrecanase activity) (Fig. 4A), (ii) the BC-3 antibody that recognizes the newly generated amino-terminal 374ARGVS sequence after aggrecanase activity in the IGD of aggrecan (Fig. 4B), and (iii) the anti-GELE antibody that detects the newly generated carboxyl-terminal GELE1480 sequence after aggrecanase activity in the CS2 region of aggrecan (Fig. 4C). Aggrecanolytic activity relative to TS4-2 (set as 100) was estimated from each Western blot, and the results are shown in Table 1.

**Analyses with the 2-B-6 antibody** showed the general pattern of aggrecan degradation by full-length ADAMTS-5 (TS5-1) and its domain deletion mutants (Fig. 4A). A comparison of the products demonstrated that TS5-1 and TS5-2 had the highest general aggrecanase activity with TS5-2 consistently showing slightly higher activity. This activity was drastically reduced by the removal of the Sp domain (TS5-3) and further decreased by the removal of the CysR (TS5-4) and the first TS (TS1) domains (TS5-5). Aggrecan degradation was never detected with the catalytic domain alone (TS5-6). It is notable that the aggrecan digestion pattern of TS5-4 is different from that of TS5-3, indicating that the CysR and Sp domains influence both specificity and specific activity. Comparison of product formation between ADAMTS-4 and -5 indicated that ADAMTS-5 was about 1,000 times more active than ADAMTS-4. In addition, ADAMTS-4 generated an additional 200-kDa band (Fig. 4A, la) that was not present in the ADAMTS-5 digests, indicating that ADAMTS-4 cleaves aggrecan at a unique site.

**Analyses using the BC-3 antibody** showed that TS5-1 is most active in cleaving the Glu373-Ala374 bond located in the IGD (Fig. 4B). Deletion of the carboxyl-terminal TS2 domain reduced the activity by...
about 2-fold. A further 2-fold reduction may be observed with deletion of the Sp domain if we consider the 0.5-h time point of TS5-2 to be similar to the 1-h time point of TS5-3. Subsequent deletion of the CysR domain (TS5-4) caused a further 50-fold decrease in activity. TS5-4 was 3-fold more active than TS4-2.

No activity was detected using 1 nM TS5-5 or TS5-6. TS5-3, which contains a domain structure similar to that of TS4-2, was about 150-fold more active than TS4-2 at this site.

When the digestion products were analyzed with the anti-GELE1480 antibody (Fig. 4C), TS5-1 and TS5-2 showed the highest activity and cleaved the Glu1480-Ala1481 bond to a similar extent. This activity sharply decreased (roughly to 1/100) with TS5-3, indicating that the Sp domain is crucial for the cleavage at this site. The activity was further reduced after removal of the CysR domain. No activity was detected with TS5-5 and TS5-6. TS5-3, which contains a domain structure similar to that of TS4-2, was about 150-fold more active than TS4-2 at this site.

When the digestion products were analyzed with the anti-GELE1480 antibody (Fig. 4C), TS5-1 and TS5-2 showed the highest activity and cleaved the Glu1480-Ala1481 bond to a similar extent. This activity sharply decreased (roughly to 1/100) with TS5-3, indicating that the Sp domain is crucial for the cleavage at this site. The activity was further reduced after removal of the CysR domain. No activity was detected with TS5-5 and TS5-6. TS5-3, which contains a domain structure similar to that of TS4-2, was about 150-fold more active than TS4-2 at this site.

The aggrecanase activity of ADAMTS-4 is greatly reduced when aggrecan is deglycosylated (17, 31). We therefore digested native and deglycosylated aggrecan with 0.01 nM TS5-1 and 5 nM TS4-2. As is shown in Fig. 5, the aggrecanase activity of ADAMTS-4 and -5 at the GELE1480 site was drastically reduced when the aggrecan was deglycosylated. Similar results were obtained when the samples were probed using the 2-B-6 antibody (data not shown). Nonetheless TS5-1 was at least 500-fold more active than TS4-2 on deglycosylated aggrecan.

Activity against Cm-Tf—Full-length ADAMTS-4 has little general proteolytic activity, but deletion of the Sp domain reveals the activity against Cm-Tf (17). However, unlike ADAMTS-4, full-length ADAMTS-5 (TS5-1), TS5-2, and TS5-3 degraded Cm-Tf to a similar extent (Fig. 6A and Table 1), indicating that deletion of the carboxyl-terminal TS1 domain and the Sp domain does not affect its activity. The deletion of the CysR domain (TS5-4) caused about 4-fold decrease in this activity. A further 2-fold reduction in activity was seen with the deletion of the TS1 domain (TS5-5), but TS5-6 showed very little activity even at concentrations as high as 100 nM. Comparison of TS4-2 and TS5-1 indicated that TS5-1 was about 2-fold more active on Cm-Tf. Inhibition of the Cm-Tf activity...
by N-TIMP-3, but not by N-TIMP-1 or TIMP-2 (Fig. 6B), indicates that this activity is due to the direct action of ADAMTS-5.

Cm-Tf digestion patterns revealed that full-length ADAMTS-5 and its domain deletion mutants have similar specificity, but they were different from those of ADAMTS-4 (Fig. 7A), suggesting that substrate specificity between the two enzymes is not identical. Amino-terminal sequencing of the fragments generated by TS5-4 indicated that all of its cleavage sites were also cleaved by TS4-2 but that ADAMTS-5 recognized a fewer number of cleavage sites. As summarized in Fig. 7B, ADAMTS-5 cleaves Cm-Tf at three major sites and one minor site, whereas TS4-2 has many additional sites.

Fibromodulin Digestion—TS5-1, TS5-2, and TS5-3 digested fibromodulin in a manner similar to that of Sp-truncated TS4-2 (Fig. 8). The further deletion of the CysR domain (TS5-4), the TS1 domain (TS5-5), and the Dis domain (TS5-6) progressively worsened the ability of TS5 to cleave fibromodulin. These results were similar to those observed for Cm-Tf. Longer incubation times with a higher concentration of ADAMTS-4 or ADAMTS-5 catalytic domain demonstrated that, although both enzymes have very low activity toward fibromodulin (Fig. 8B), the ADAMTS-5 catalytic domain (TS5-6) was far more active than the ADAMTS-4 catalytic domain (TS4-5). The amino terminus of the 29-kDa fibromodulin fragment generated by ADAMTS-5 was 45AYGSP, indicating that cleavage occurred at the Tyr44-Ala45 bond, an identical site cleaved by ADAMTS-4 (17) and MMP-13 (32).

Other Substrates—Other small leucine-rich repeat proteoglycans, decorin and biglycan, were also degraded by ADAMTS-5. A comparison between ADAMTS-4 and -5 suggested that decorin was a slightly better substrate for ADAMTS-4, whereas biglycan was better for ADAMTS-5 (Fig. 9). Both enzymes degraded fibronectin but at a very slow rate (Fig. 9). Domain deletion mutants of ADAMTS-5, with the exception of TS5-6, also degraded fibronectin (data not shown). In addition, both ADAMTS-4 and -5 digested casein, but their gelatinolytic activity was only weakly detected (data not shown).

pH Optimum of ADAMTS-4 and ADAMTS-5

The pH-dependent activities of ADAMTS-4 and ADAMTS-5 were examined using aggrecan and Cm-Tf as substrates. Both enzymes exhibited a broad optimal activity against aggrecan encompassing pH 7.0–9.0 (Fig. 10A and B). A similar pH optimal activity was detected when Cm-Tf was used as substrate (Fig. 10C and D).

Salt Effects on Proteolytic Activity of ADAMTS-4 and ADAMTS-5

We postulated that the ionic strength of the solution may affect aggrecanase activity by altering the interaction between the enzyme and the negatively charged glycosaminoglycan moieties of the aggrecan substrate. To test this possibility, we digested aggrecan with ADAMTS-4 or ADAMTS-5 in various concentrations of NaCl.

As shown in Fig. 11A, the aggrecanase activity of TS4-2 was greatly affected by the presence of NaCl. The highest activity was detected with 12.5–50 mM NaCl, but it rapidly dropped when the salt concentration was above 50 mM. At 150 mM NaCl, only about 20% of the maximal activity was detected; essentially no activity was detected at or above 200 mM NaCl.

The aggrecanase activity of ADAMTS-5 was also drastically affected by NaCl but differently from the effect on ADAMTS-4. This activity was very low at or below 50 mM NaCl or above 500 mM NaCl. Optimal aggrecanase activity for ADAMTS-5 was detected around 200 mM NaCl, and about 80% of its activity was retained at 150 mM NaCl. In contrast to their aggrecanase activ-
ities, the general proteolytic activities of ADAMTS-4 and -5, as measured with Cm-Tf, were unaffected by concentrations up to 1 M NaCl (Fig. 10B shows the results up to 500 mM NaCl). These results indicate that the interaction between the ancillary domains of the two ADAMTSs and polyanionic polysaccharides of aggrecan greatly influences their aggrecanolytic activities, whereas their general proteolytic activities are independent of ionic interactions.

**DISCUSSION**

Accumulating evidence indicates that the proteolytic activities of multidomain ADAMTS metalloproteinases on natural substrates are regulated by their non-catalytic ancillary domains. For example, the N-procollagen endopeptidase activity of ADAMTS-2 is negatively regulated by its carboxyl-terminal domain specific to N-procollagen endopeptidases but positively regulated by its three carboxyl-terminal TS domains (33). The carboxyl-terminal eight TS domains and two CUB domains of ADAMTS-13 are dispensable for its von Willebrand factor cleaving activity, but the Sp domain plays a critical role in binding of the enzyme to von Willebrand factor and cleavage (34–37). Furthermore carboxyl-terminally truncated forms of ADAMTS-1 (38), ADAMTS-8 (39), ADAMTS-9 (40), and ADAMTS-20 (41) are largely inactive against natural substrates. The carboxyl-terminal CysR and Sp domains of ADAMTS-4 play an important role in regulating its proteolytic activity (17, 42). In this study, we examined the role of ADAMTS-5 carboxyl-terminal domains using an approach similar to that used in our ADAMTS-4 study (17). Successful purification of each ADAMTS-5 domain deletion mutant, as well as quantification of the active enzyme amount by N-TIMP-3, enabled us to characterize how each domain contributes to the proteolytic activities of ADAMTS-5. Furthermore we were able to compare these activities with ADAMTS-4. Although the data presented in Table 1 are estimates of relative activities due to the limitation of currently available assay methods, they show a number of key differences in enzymatic properties between the two aggrecanases.

The most striking observation is that full-length ADAMTS-5 was ~1,000-fold more active in degrading aggrecan than ADAMTS-4 under physiological conditions. Previously Tortorella et al. (21) reported that ADAMTS-5 is a weaker aggrecanase than ADAMTS-4. On the other hand, Roughley et al. (43) reported that the cleavage within the IGD of fetal bovine and neonatal human aggrecans by ADAMTS-5 is more pronounced than that by ADAMTS-4 but that the activities of the two aggrecanases are similar on adult bovine and human aggrecans. Discrepancies between our study and these reports may be due to differences in the purity of the enzymes used and/or how the enzymes were handled. All these factors would greatly influence their apparent activity as auto-processing of both ADAMTS is often observed (17, 42, 44). Other major differences include how the ancillary domains of ADAMTS-4 and ADAMTS-5 influenced their proteolytic activities. For example, the Sp domain of ADAMTS-4 largely masked its activity on the “classical” aggrecanase site (the Glu373-Ala374 bond in the IGD) and its general proteolytic activity. However, the Sp domain of ADAMTS-5 did not inhibit any of its proteolytic activities. Removal of the CysR domain appeared to have a minor influence on ADAMTS-4 activity (about 2-fold reduction in activity) but a much larger reduction of ADAMTS-5 activity depending on the substrate (Table 1). The removal of both CysR and Sp domains from ADAMTS-5

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6 C. Gendron, unpublished observation.
Proteolytic Activities of ADAMTS-5

(TS5-4) greatly reduced its aggrecanolytic activity to 0.2-0.01% of the full aggrecanolytic activity of TS5-1. In contrast, it had little influence on the hydrolysis of Cm-Tf. Removal of the CysR and Sp domains (TS5-4) also led to the release of enzymes into the medium without heparin addition (Fig. 3). These results suggest that CysR and Sp domains may interact with the sulfated polysaccharide chains of aggrecan and heparan sulfate proteoglycans on the cell surface or in the ECM. This notion is supported by the fact that removal of the CysR and Sp domains (TS5-4) led to the release of enzyme into the medium without heparin addition (Fig. 3). Regardless of these changes, the general aggrecanase activity of TS5-4 was still about 3 times more active than that of TS4-2. Further removal of the first TS and Dis domains of ADAMTS-5 drastically reduced its proteolytic activity like ADAMTS-4 (17), and the catalytic domain of ADAMTS-5 was essentially inactive. Nevertheless the catalytic domain of ADAMTS-5 (TS5-6) exhibited the ability to cleave fibromodulin after a long incubation, and this activity was about 25 times greater than that of the catalytic domain of ADAMTS-4. This suggests that there are fundamental differences between the two catalytic domains, but the structural basis for this difference is not clear at the moment.

In the tissue, ADAMTS-4 is processed by MT4-MMP (MMP-17) on the cell surface, an event that leads to the removal of the carboxyl-terminal Sp domain (19). In IL-1-stimulated cartilage, we observed a 46-kDa form of ADAMTS-4 (17). More recently, an alternatively spliced ADAMTS-4 mRNA that lacks most of the Sp domain but instead encodes a new unrelated carboxyl-terminal sequence has been reported in human synovium from osteoarthritic patients (45). These ADAMTS-4 variants, which result from alternative mRNA splicing or posttranslational processing, are likely to express altered enzymatic activities. We attempted to identify the forms of ADAMTS-5 in pig articular cartilage stimulated with IL-1, but detection of ADAMTS-5 protein was not successful. This is probably due to low protein levels of ADAMTS-5 in IL-1-treated cartilage. Taking the specific activity of ADAMTS-4 on aggrecan that we reported previously (34 mg of glycosaminoglycan is degraded by 1 nmol of ADAMTS-4/min at 37 °C) that we reported previously (17), we estimated that only 50 pg of ADAMTS-5 is sufficient to degrade all of the aggrecan in 1 g of cartilage in a 2-day cartilage explant culture, assuming that the degradation is all due to ADAMTS-5. Our Western blot analysis to detect ADAMTS-5 required at least 1 ng of the enzyme. This indicates that at least 20 g of cartilage need to be processed to detect ADAMTS-5 for a single Western blot analysis. Nonetheless there are a few studies reporting Western blot analyses of ADAMTS-5 in the medium of human synovial cells (20, 46) and in human osteoarthritis cartilage (47) using Western blot analysis.

Similar to ADAMTS-4 (17, 31), the sulfated polysaccharide chains on aggrecan greatly influence the aggrecanase activity of ADAMTS-5. The ionic nature of the interaction of ADAMTS-4 or -5 with sulfated glycosaminoglycans is supported by their sensitivity to NaCl concentration. In this regard, ADAMTS-4 was much more sensitive to physiological salt concentrations than ADAMTS-5. The reason for the relatively little activity of ADAMTS-5 against aggrecan at low NaCl concentrations is presently unclear, but it may be due to structural changes within the enzyme itself or to increased unproductive interactions between ADAMTS-5 and the substrate. Nonetheless the........
activity of ADAMTS-5 on deglycosylated aggrecan was about 500 times stronger than that of ADAMTS-4 under the physiological salt concentration. Thus, differences in aggrecanase activity between ADAMTS-4 and -5 may lie in both the intrinsic activity of their catalytic domains and their affinities for aggrecan.

Prior to our characterization of ADAMTS-4 activities on Cm-Tf and fibromodulin (17), it had been considered that aggrecanases had a requirement for glutamic acid in the P1 site (21, 48). TS4-2 digests a peptide bond after methionine, leucine, serine, glycine, aspartic acid, and carboxymethylated cysteine in Cm-Tf, but its actions on those residues are highly restricted. Because the sequences around the sessile bonds are promiscuous, we propose that the enzyme recognizes certain secondary structures around the cleavage site (17). A similar observation was made for ADAMTS-5, although in this case its specificity on Cm-Tf was even more restricted (Fig. 7). Such selectivity is likely to arise from either the catalytic domain itself or the Dis domain because the pattern of Cm-Tf digestion was similar for all domain deletion mutants of ADAMTS-5 (the catalytic domain alone was unable to degrade Cm-Tf, thereby making it impossible to determine whether it alone was responsible).

We also showed that ADAMTS-4 and -5 are able to degrade decorin, biglycan, fibrocetin, fibronectin, and gelatin. However, it should be noted that fibronectin and gelatin were very poor substrates. Their degradation products were only visible after long incubation times. Thus, the biological and pathological relevance of these minor activities is questionable. Nevertheless, the identification of new, cartilage-specific substrates for the two aggrecanases suggests that they may have a broader role in cartilage matrix degradation than was reported initially. Fibromodulin fragmentation has been shown to occur within the 1st week of IL-1 treatment (32). Recent studies of Melching et al. (50) showed that both ADAMTS-4 and -5 cleave biglycan at the Asn149-Cys150 bond, and cleaved biglycan products were found in human articular cartilage from patients with osteoarthritis and rheumatoid arthritis. They also reported that these two aggrecanases did not cleave decorin. As shown in this work the ability of ADAMTS-4 and TSS to cleave decorin is weaker than their ability to cleave biglycan. Although aggrecan is a substrate very sensitive to ADAMTS-4 and -5, degradation of other matrix components by these proteinases in diseases such as arthritis may lead to further destabilization of the articular cartilage matrix.

Our data, in combination with recently published results, support a more prominent role for ADAMTS-5 than for ADAMTS-4 in the progression of arthritis. Although research comparing the mRNA levels of ADAMTS-4 and -5 in normal and osteoarthritic human cartilage explants have yielded inconsistent results (12, 51), these studies consistently found that ADAMTS-5 expression is higher than ADAMTS-4. Additional evidence for a reduced role for ADAMTS-4 in osteoarthritis comes from work using transgenic animal models. Mice lacking ADAMTS-4 develop normally and develop surgically induced osteoarthritis in a manner similar to that in wild-type mice (13), suggesting that lack of ADAMTS-4 does not protect against the disease. However, deletion of ADAMTS-5 protects mice from aggrecan loss and cartilage erosion in two different arthritis models (15, 16). Spontaneous aggrecan degradation is also ablated in murine epiphyseal chondrocyte cultures of ADAMTS-5-null mice as compared with cultures from wild-type mice (52). Although it is still not clear which proteinase plays the major role in aggrecanolysis during the development of arthritis in humans, this work reveals that ADAMTS-5 is a strong contender.

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Proteolytic Activities of ADAMTS-5

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