Functional Differences of the Catalytic and Non-catalytic Domains in Human ADAMTS-4 and ADAMTS-5 in Aggrecanolytic Activity*

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ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) are multidomain metalloproteinases belonging to the ADAMTS family. We have previously reported that human ADAMTS-5 has much higher aggrecanolytic activity than human ADAMTS-4. To investigate the different proteolytic activity of the two enzymes, we generated a series of chimeras by exchanging various non-catalytic domains of the two proteinases. We found that the catalytic domain of ADAMTS-5 has higher intrinsic catalytic ability than that of ADAMTS-4. The studies also demonstrated that the non-catalytic domains of ADAMTS-5 are more effective modifiers than those of ADAMTS-4, making both catalytic domains more active against aggrecan, an Escherichia coli-expressed interglobular domain of aggrecan and fibromodulin. Addition of the C-terminal thrombospondin type 1 motif of ADAMTS-5 to the C terminus of ADAMTS-4 increased the activity of ADAMTS-4 against aggrecan and fibromodulin severalfold. In contrast to previous reports (Kashiwagi, M., Enghild, J. J., Gendron, C., Hughes, C., Caterson, B., Itoh, Y., and Nagase, H. (2004) J. Biol. Chem. 279, 10109–10119 and Gao, G., Plaa, A., Thompson, V. P., Jin, S., Zuo, F., and Sandy, J. D. (2004) J. Biol. Chem. 279, 10042–10051), our detailed investigation of the role of the C-terminal spacer domain of ADAMTS-4 indicated that full-length ADAMTS-4 is ~20-times more active against aggrecan than its spacer domain deletion mutant, even at the Glu373-Ala374 site of the interglobular domain. This discrepancy is most likely due to selective inhibition of full-length ADAMTS-4 by heparin, particularly for cleavage at the Glu373-Ala374 bond. However, removal of the spacer domain from ADAMTS-4 greatly enhanced more general proteolytic activity against non-aggrecan substrates, e.g. E. coli-expressed interglobular domain, fibromodulin, and carboxymethylated transferrin.

A common feature of osteoarthritis (OA)2 and rheumatoid arthritis is destruction of articular cartilage, which is characterized by a homeostatic imbalance between synthesis and degradation of the extracellular matrix (ECM). The destructive process is believed to be due to elevated activities of proteolytic enzymes that degrade macromolecules of the cartilage ECM such as aggrecan and type II collagen fibrils. Aggrecan is the major proteoglycan in articular cartilage, and it forms large aggregates by interacting with hyaluronan and link protein. Aggrecan monomers consist of a core protein with chondroitin sulfate (CS) and keratan sulfate (KS) polysaccharide chains. The core protein consists of several segments, including an N-terminal globular domain (G1), an interglobular domain (IGD), a second globular domain (G2), a long glycosaminoglycan (GAG) attachment region, including KS-rich and CS-rich (CS-1 and CS-2) regions, and a C-terminal globular domain (G3) (1). Aggrecans are highly hydrated because of their long negatively charged polysaccharide chains. Thus, within the collagen framework, they enable the cartilage to resist mechanical compression as a load-bearing surface. Aggrecan loss is therefore considered to be a crucial initial event in the development of arthritis, which is followed by essentially irreversible collagen degradation (2, 3). This pathological aggrecan degradation in articular cartilage is driven mainly by proteolytic enzymes termed aggrecanases and matrix metalloproteinases (MMPs) (4, 5).

The aggrecanases are members of the ADAMTSSs (a disintegrin and metalloproteinase with thrombospondin type 1 motifs) family (6). The first two proteinases identified to cleave the aggrecan core protein at the aggrecanase-specific Glu373-Ala374 bond in the IGD were ADAMTS-4 (aggrecanase-1) (7) and ADAMTS-5 (aggrecanase-2) (8). Finding aggrecan fragments cleaved at the Glu373-Ala374 bond in synovial fluids and cartilage from patients with OA and rheumatoid arthritis suggested that aggrecanases play an important role in cartilage destruction (9, 10). They are also the primary enzymes that cleave aggrecan in response to inflammatory cytokines in articular cartilage explant systems (11). Gendron et al. (12) have shown that cleavage of the Glu373-Ala374 bond in interleukin-1-stimulated cartilage explants is blocked by tissue inhibitor of metalloproteinases-3 (TIMP-3), but not by other TIMPs, indicating that ADAMTSSs play the major role in aggrecan degradation in this model of cartilage degradation.

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Among the ADAMTS enzymes, ADAMTS-4 and -5 have received most attention in the pathology of arthritic joint diseases, because they are the most efficient aggrecanases in vitro (13, 14). Other ADAMTSs, such as ADAMTS-1, -8, -9, -15, -16, and -18 have been shown to cleave aggrecan, but they have very weak activity (6, 15). Furthermore, recent studies have shown that mice lacking ADAMTS-5, but not ADAMTS-4 (16) or ADAMTS-1 (17), are significantly protected against aggrecan degradation in inflammatory (18) or surgically induced models of arthritis (19). This suggests that ADAMTS-5 plays a key role in aggrecan degradation, at least in these mouse models of arthritis. On the other hand, Song et al. (20) recently reported that a reduction in both ADAMTS-4 and -5 expression was required to inhibit aggrecan degradation in human OA cartilage, suggesting that both ADAMTSs may play a role in human OA. Therefore, it is still not clear which aggrecanase plays the main role in pathological cartilage degradation in humans.

ADAMTS-4 and -5 are multidomain metalloproteinases belonging to the adamalysin subfamily of the metalloclopeptidase M12 family (21). Both enzymes have a similar domain arrangement, consisting of a pro-domain, a catalytic metalloproteinase domain, a disintegrin-like (Dis) domain, a thrombospondin type I (TS) domain, a cysteine-rich (CysR) domain and a spacer (Sp) domain. In addition, ADAMTS-5 contains an additional TS domain after the Sp domain (22) (see Fig. 1). We have previously shown that the non-catalytic ancillary domains of ADAMTS-4 and -5 play a major role in regulating aggrecanase activity and substrate specificity (13, 14). Furthermore, Flannery et al. (23) showed that the CysR and Sp domains of ADAMTS-4 are necessary for the enzyme’s interaction with the GAG chains of aggrecan, and Tortorella et al. (24) reported that the TS domain of ADAMTS-4 is critical for aggrecan recognition and cleavage. However, the roles of the non-catalytic domains of ADAMTS-4 and -5 are not completely understood at present.

Our recent studies showed that the aggrecanolytic activity of ADAMTS-5 was much greater than ADAMTS-4 in both the IGD and the CS-2 regions (13). This provided biochemical evidence supporting the view that ADAMTS-5 is the major aggrecanase in cartilage catabolism and pathology. The study also revealed that the roles of the non-catalytic ancillary domains of the two enzymes in aggrecan digestion are significantly different. In the case of ADAMTS-4, it was shown that full-length enzyme (70 kDa) was the most effective aggrecanase, but it exhibited little activity against the Glu373-Ala374 bond (14, 25). When the Sp domain was removed by MT4-MMP (MMP-17) or mutagenesis, the resulting 53-kDa form of ADAMTS-4 exhibited effective hydrolysis of aggrecan at the IGD site (14, 26). The 53-kDa form also showed new proteolytic activities against carboxymethylated transferrin (Cm-Tf), fibromodulin, and decorin (14), suggesting that the Sp domain regulates certain proteolytic activities of ADAMTS-4. In contrast, the Sp domain of ADAMTS-5 does not exhibit any such regulatory activities. Further removal of CysR domain from ADAMTS-4 has a relatively minor influence on aggrecanolytic activity, but the removal of both CysR and Sp domains from ADAMTS-5 greatly reduces its aggrecanolytic activity. When the TS and Dis domains were further removed from both aggrecanases, the catalytic metalloproteinase domains alone were not active. Nonetheless, the structural basis for ADAMTS-5 being a much more efficient aggrecanase than ADAMTS-4 has not been elucidated.

The aim of this study is to investigate the functional difference in the catalytic and C-terminal domains of ADAMTS-4 and ADAMTS-5 and to understand the reason(s) behind their extremely different proteolytic activities. For this purpose, we designed chimeras in which various domains were exchanged between ADAMTS-4 and ADAMTS-5 and measured their enzymatic activities against native bovine aggrecan, an Escherichia coli-expressed recombinant protein substrate containing the IGD of aggrecan, and fibromodulin. We also re-assessed the proteolytic activities of full-length ADAMTS-4 and C-terminal spacer-domain-truncated ADAMTS-4.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney (HEK293-EBNA) cells, pCEP4 plasmid vector, and pMT/V5HisA vector were from Invitrogen. Restriction enzymes, T4 DNA ligase, and peptide N-glycosidase F (N-glycosidase F) were from New England Biolabs (Hitchin, UK). Pfu Turbo DNA polymerase was from Stratagene Europe (Amsterdam, Netherlands). FuGENE6 transfection reagent was from Roche Applied Science. Alkaline phosphatase-conjugated goat anti-(mouse IgG) IgG, alkaline phosphatase-conjugated goat anti-(rabbit IgG) IgG, and Western Blue-stabilized substrate for alkaline phosphatase were from Promega (Southampton, UK). Anti-FLAG M2 antibody (mouse monoclonal antibody), anti-FLAG M2-agarose, FLAG peptide, heparin, and human apo-transferrin were from Sigma–Aldrich. Chondroitinase ABC and keratanase were from Seikagaku Kogyo (Tokyo, Japan). Sephacryl S-200 was from Amersham Biosciences. Macro-Prep 25 S resin and pre-stained and unstained Precision Protein Standards for SDS-PAGE were from Bio-Rad. PD-10 desalting columns were from GE Health-care (Bucks, UK). Vivaspin centrifugal filter units were from Sartorius (Goettingen, Germany). S- Carboxymethylated transferrin (Cm-Tf) was prepared according to method of Nagase (27). Aggrecan monomers were purified from bovine nasal cartilage under dissociative conditions according to Hascall and Sajdera (28). The N-terminal domain of human TIMP-3 with C-terminal His tag (N-TIMP-3) was expressed in E. coli, folded in vitro, and purified (29). The monoclonal antibody BC-3 (anti-ARGS) that recognizes the N-terminal neoepitope 374 ARGSV of aggrecan core protein was kindly supplied by Dr. Clare Hughes and Prof. Bruce Caterson (University of Cardiff, Cardiff, UK). The rabbit anti-GELE antibody that recognizes the new GELE1480 C terminus generated by aggrecanase cleavage of the aggrecan core protein has been previously described (14). The rabbit anti-human ADAMTS-4 catalytic domain and anti-human ADAMTS-5 catalytic domain antibodies were raised in rabbits and purified as described previously (13, 14). All other reagents used were of the highest analytical grade available.

Construction of cDNA Coding for ADAMTS-4, ADAMTS-5, and ADAMTS Chimeras—cDNA encoding human ADAMTS-4 and ADAMTS-5 were obtained as described (13, 14), and FLAG (DYKDDDDK)-tagged ADAMTS-4-1 (full-length ADAMTS-
Domain Functions in ADAMTS-4 and -5

4) and ADAMTS-5 were constructed as previously described (13, 14) using the 5' forward primer and the 3' reverse primers: 5'-ATGTTCCAGACAGGCTGATCCCGGAGG-3' (ADAMTS4/FW) and 5'-CTAATCTTTCGGCTCATCTTGGTACTCCCGCCAGG-3' (ADAMTS5/FLAG/RV). The ADAMTS-4 sequence is shown in Fig. 1 and was constructed using wild-type ADAMTS-4 and wild-type ADAMTS-5 cDNAs as templates for overlapping polymerase chain reactions. For KF1, -2, and -3, using the ADAMTS4/FW primer and an antisense chimeric junction primer containing the ADAMTS-5 sequence at the 5' and the ADAMTS-4 sequence at the 3', the N-terminal part of the chimer containing primarily the ADAMTS-4 sequence was made. Using ADAMTS5/FLAG/RV and a sense chimeric junction primer containing the ADAMTS-4 sequence at the 5' and the ADAMTS-5 sequence at the 3' (overlapping with the previous primer), the C-terminal part of the chimer containing primarily the ADAMTS-5 sequence was made. A final PCR was performed using the two flanking primers (ADAMTS4/FW and ADAMTS5/FLAG/RV) and these two first round products as the template. For KF4 and -5, using the sense ADAMTS5/FW primer and an antisense chimeric junction primer containing the ADAMTS-4 sequence at the 5' and the ADAMTS-5 sequence at the 3', the N-terminal part of the chimer containing primarily the ADAMTS-4 sequence was made. Using ADAMTS5/FLAG/RV and a sense chimeric junction primer containing the ADAMTS-4 sequence at the 5' and the ADAMTS-5 sequence at the 3' (overlapping with the previous primer), the C-terminal part of the chimer containing primarily the ADAMTS-5 sequence was made. Using ADAMTS5/FLAG/RV and a sense chimeric junction primer containing the ADAMTS-4 sequence at the 5' and the ADAMTS-5 sequence at the 3', the N-terminal part of the chimer containing primarily the ADAMTS-4 sequence was made. A final PCR was performed using these two first round products as the template and the two flanking primers (ADAMTS5/FW and ADAMTS4/FLAG/RV). The final reaction resulted in a full-length ADAMTS chimera shown in Fig. 1 and was constructed using wild-type ADAMTS-4 and wild-type ADAMTS-5. The ADAMTS chimeras shown in Fig. 1 were constructed using wild-type ADAMTS-4 and wild-type ADAMTS-5. Each protein contains a FLAG epitope (DYKDDDDK) at its C terminus. Signal peptide and pro-domain are not included in this figure, because they were processed intracellularly and recombinant proteins purified were active forms. Cat, catalytic domain; Dis, disintegrin-like domain; TS, thrombospondin type I domain; CysR, cysteinereich domain; and Sp, spacer domain.

FIGURE 1. Schematic representation of ADAMTS-4/ADAMTS-5 chimeras. The ADAMTS-4 sequence is shown in white, and ADAMTS-5 sequence is shown in gray. Key amino acids are labeled to indicate the first and last residues of ADAMTS-4 and ADAMTS-5. Each protein contains a FLAG epitope (DYKDDDDK) at its C terminus. Signal peptide and pro-domain are not included in this figure, because they were processed intracellularly and recombinant proteins purified were active forms. Cat, catalytic domain; Dis, disintegrin-like domain; TS, thrombospondin type I domain; CysR, cysteine-rich domain; and Sp, spacer domain.
the Macro-Prep 25 S resin under these conditions. The column was washed with 50 mM Tris-HCl (pH 7.5) containing 250 mM NaCl, 10 mM CaCl₂, 0.02% Brij-35, and 0.02% NaN₃, and recombinant protein was eluted with 50 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 10 mM CaCl₂, 0.02% Brij-35, and 0.02% NaN₃. Finally, the buffer was exchanged for aggrecanase reaction buffer using a PD-10 desalting column. For purification of KF1, a Macro-Prep 25 S resin was also used to remove the FLAG peptide and contaminants. After application onto a Macro-Prep 25 S resin, the column was washed with 50 mM Tris-HCl (pH 7.5) containing 2 M NaCl, 10 mM CaCl₂, 0.02% Brij-35, and 0.02% NaN₃. The protein was eluted with 50 mM Tris-HCl (pH 7.5) containing 3 M urea, 2 M NaCl, 10 mM CaCl₂, 0.02% Brij-35, and 0.02% NaN₃. Finally, the buffer was exchanged for aggrecanase reaction buffer using a PD-10 desalting column. The purity of recombinant ADAMTSs was confirmed by SDS-PAGE and Western blotting analysis using anti-ADAMTS-4 catalytic domain, anti-ADAMTS-5 catalytic domain, or anti-M2-FLAG antibodies. The concentrations of active recombinant ADAMTSs were determined by titration with known concentrations of N-TIMP-3.

SDS-PAGE and Western Blotting—SDS-PAGE was carried out under reducing conditions (30), and proteins were stained by silver (31). For Western blotting analyses, proteins separated by SDS-PAGE were electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline and then incubated with primary antibodies recognizing FLAG M2 (1:2000 dilution), ADAMTS-4 catalytic domain (1:1000 dilution), ADAMTS-5 catalytic domain (1:1000 dilution), BC-3 neoepitope (1:200 dilution), or GELE1480 neoepitope (1:2000 dilution). The blot was then incubated with secondary antibody conjugated to alkaline phosphatase, and the protein bands were visualized using Western Blue stabilized substrate.

Aggrecanase Assays—To quantify the aggrecan-degrading activity of the various ADAMTS forms, bovine aggrecan (750 nm) was incubated with ADAMTS-4, ADAMTS-5, or chimera in aggrecanase reaction buffer at 37 °C. The reactions were terminated with 50 mM EDTA, and the digestion products were deglycosylated overnight at 37 °C with chondroitinase ABC (0.01 unit/10 μg of aggrecan) and keratanase (0.01 unit/10 μg of aggrecan) in 100 mM Tris-HCl (pH 8.0), 100 mM sodium acetate, and 50 mM EDTA. The aggrecan core protein was precipitated with 5 volumes of acetone, and incubated at −20 °C for 1 h. The pellet was dried and dissolved in reducing SDS sample buffer. The products were subjected to SDS-PAGE before Western blot analysis with the BC-3 or anti-GELE antibody as primary antibodies. For comparative studies, all polyvinylidene difluoride membranes were processed simultaneously and Western analyses were carried out under identical conditions. Aggrecan samples digested by ADAMTS-4 were used as a standard, and always applied on the same gel or blot as the other ADAMTS forms. Gels and blots were scanned using a Bio-Rad GS-710 scanning densitometer (Hemel Hempstead, UK), and the band intensity was quantified using one-dimensional Phoretix quantification software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Recombinant IGD Substrate Digestion Assay—A recombinant aggrecanase substrate containing the IGD sequence flanked by glutathione S-transferase at the N terminus and FLAG peptide at the C terminus (gst-IGD-flag) was expressed in E. coli and purified using glutathione-Sepharose.³ gst-IGD-flag (17 μM) was incubated with 10 nm of various ADAMTS enzymes in aggrecanase reaction buffer at 37 °C for 15 min to 4 h. The reaction was terminated with 20 mM EDTA, and the products were analyzed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining. The amount of the product was quantified by densitometric analysis using ID Phoretix quantification software.

Cm-Tf and Fibromodulin Digestion Assay—Cm-Tf (2.5 mg/ml) was incubated with 10 nm of ADAMTSSs in aggrecanase reaction buffer at 37 °C for 4–24 h, and the products were analyzed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining. Fibromodulin (0.1 mg/ml) was reacted with ADAMTSs in aggrecanase reaction buffer. The reaction was terminated with 20 mM EDTA, and the products were deglycosylated using N-glycosidase F (250 units/1 μg of fibromodulin). The products were analyzed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining.

RESULTS

Expression of the Recombinant ADAMTSs—Full-length ADAMTS-4 (ADAMTS-4-1), the C-terminal spacer domain deletion mutant of ADAMTS-4 (ADAMTS-4-2), full-length ADAMTS-5, and chimeras were purified from the conditioned medium of stably transfected HEK293-EBNA cells. Because the Sp domain of ADAMTS-4 and the Cys-R domain of ADAMTS-5 play significant roles in ECM binding (13, 14), 100 μg/ml heparin was added to the medium to prevent the enzymes from binding to the cell layer and ECM components. We successfully purified each ADAMTS form and determined the active enzyme concentration by titration with N-TIMP-3. All preparations were homogenous on silver-stained SDS-PAGE (Fig. 2A). Purified enzymes were also detected by Western blotting analysis with an anti-FLAG M2 antibody (Fig. 2B), anti-ADAMTS-4 catalytic domain (Fig. 2C), and anti-ADAMTS-5 catalytic domain antibodies (Fig. 2D). Approximately 50 μg of ADAMTS-4-1, 20 μg of KF1, 90 μg of KF2, 80 μg of KF3, 20 μg of KF4, 20 μg of KF5, and 60 μg of ADAMTS-5 were obtained per liter of conditioned medium. The molecular mass of the recombinant proteins was measured by SDS-PAGE to be 69 kDa for ADAMTS-4-1, 75 kDa for KF1, 80 kDa for KF2, 80 kDa for KF3, 69 kDa for KF4, 72 kDa for KF5, and 81 kDa for ADAMTS-5. Measured molecular masses indicated that the propeptides of all enzymes were processed intracellularly. However, the molecular masses of the KF2, KF3, KF5, and ADAMTS-5 were slightly higher than predicted from their amino acid compositions. The predicted molecular masses of KF2, KF3, KF5, and ADAMTS-5 were 73, 73, 69, and 74 kDa, respectively. This difference is possibly due to glycosylation, because potential N-glycosylation sites (1 in the ADAMTS-5-Dis domain, 1 in the ADAMTS-5-CysR domain, and 2 in the

³ N. H. Lim and H. Nagase, manuscript in preparation.
ADAMTS-5-Sp domain) and O-glycosylation sites are present in the C-terminal domains of ADAMTS-5 (13).

Aggrecanase Activity of ADAMTS Chimeras—The aggrecan-degrading activities of recombinant ADAMTSs were investigated using neoepitope antibodies, which recognize either the N-terminal 374ARGS-neoepitope representing cleavage in the IGD (anti-ARGS antibody) (Fig. 3), or C-terminal GELE1480-neoepitope representing cleavage in the CS-2 region (anti-GELE antibody) (Fig. 3). The intensity of Western blot bands was quantified, and pixel volume was plotted against incubation time to determine the time dependence of aggrecan digestion. For anti-ARGS blots, a linear correlation was observed up to 4 h when aggrecan was incubated with 1 nM ADAMTS-4-1, KF1, KF2, KF3, or KF4 (data not shown). There was a linear correlation up to 1 h when aggrecan was incubated with 1 nM KF5 or ADAMTS-5 (data not shown). For anti-GELE1480 blotting, linear correlations were observed up to 4 h when aggrecan was incubated with 0.1 nM ADAMTS-4-1, KF1, or KF2, whereas plots were linear up to 1 h when aggrecan was incubated with 0.1 nM KF3, KF4, or KF5 (data not shown). Blots were linear up to 4 h when aggrecan was incubated with 0.01 nM ADAMTS-5 (data not shown). Aggrecan samples digested by ADAMTS-4-1 were used as a standard and always applied in the same gel or blot as the other ADAMTS forms. Activity was expressed relative to the activity of ADAMTS-4-1 (defined as 100) by comparing band intensity within the linear range. The results are summarized in Table 1.

Analysis of aggrecanase activity showed that ADAMTS-5 is much more active than ADAMTS-4-1 by ~30-fold in the IGD (374ARGS) and ~20-fold in the CS-2 region (GELE1480) (Fig. 3, A and B). KF1, KF2, and KF3 have the ADAMTS-4 catalytic domain functions in ADAMTS-4 and -5
TABLE 1

Relative activities of ADAMTS-4, -5, and chimeras

| Enzyme form | Aggrecan<sub>324ARGS GELE<sup>1400</sup></sub> | Recombinant substrate, <sup>gst</sup>-IGD-flag | Other substrate, fibromodulin |
|-------------|---------------------------------|---------------------------------|-------------------------------|
| ADAMTS-4-1  | 100                             | 100                             | 10                            |
| KF1         | 270                             | 400                             | 135                           |
| KF2         | 390                             | 900                             | 310                           |
| KF3         | 430                             | 900                             | 350                           |
| KF4         | 410                             | 1360                            | 730                           |
| KF5         | 810                             | 1300                            | 2670                          |
| ADAMTS-5    | 3300                            | 2200                            | 6760                          |
| ADAMTS-4-2  | 5                               | 4                               | 250                           | 100                           |

The CS-2 region (KF2). Further replacement with the Dis/1<sup>st</sup>TS/CysR/Sp domains of ADAMTS-5 (KF3) did not alter activity relative to KF2, indicating that the Dis domain of ADAMTS-5 does not affect the aggrecanolytic activity of ADAMTS-4. In general, these C-terminal domain replacements affected cleavage at the GELE<sup>1400</sup> site more than at the IGD site (KF1, -2, and -3). The reciprocal experiment was carried out by adding the ADAMTS-4 C-terminal domains to the ADAMTS-5 catalytic domain (KF4 and KF5). The addition of TS/CysR/Sp domains from ADAMTS-4 to the catalytic domain and disintegrin domain of ADAMTS-5 (KF5) reduced the activity of ADAMTS-5 by ~4-fold in the IGD and ~2-fold in the CS-2 region (KF5). Additional replacement of the Dis domain (KF4) further reduced the activity in the IGD but had little effect on cleavage in the CS-2 region (Table 1 and Fig. 3).

Proteolytic Activity against Recombinant IGD Substrate—Because KS chains of aggrecan have been shown to influence aggrecanase activity in the IGD (32, 33), the activity of the chimeras against a recombinant protein substrate, <sup>gst</sup>-IGD-flag, which lacks KS, was also investigated. Cleavage of <sup>gst</sup>-IGD-flag by aggrecanases specifically generates 32- and 17-kDa bands (Fig. 4), whereas recombinant MMP-1 generates 29- and 21-kDa bands (data not shown). By quantifying the band intensity of the specific 17-kDa product band, activity of the chimeras relative to ADAMTS-4-1 (defined as 100) was estimated (Table 1). The chimeras showed different patterns of activity against <sup>gst</sup>-IGD-flag from that observed for the IGD of aggrecan. Addition of the second TS domain of ADAMTS-5 to ADAMTS-4 (KF1) slightly increased the activity of ADAMTS-4, and further exchanging of C-terminal non-catalytic domains with those of ADAMTS-5 (KF2 and KF3) increased the activity by 3- to 3.5-fold. ADAMTS-5 was ~67-times more active than ADAMTS-4. Exchange of all of the non-catalytic domains of ADAMTS-5 with those of ADAMTS-4 (KF4) reduced the activity of ADAMTS-5 by 9-fold (Table 1). This drastic change in activity for <sup>gst</sup>-IGD-flag was similar to that for the IGD site of native aggrecan, suggesting that the C-terminal non-catalytic domains play a major role in the increased activity of ADAMTS-5 at the Glu<sup>373</sup>-Ala<sup>374</sup> bond in the IGD. In addition, KF5 was ~3.5-times more active than KF4, further suggesting that the combination of the catalytic and Dis domains of ADAMTS-5 is particularly important for the ability of ADAMTS-5 to cleave the IGD.

FIGURE 4. ADAMTS-4, -5, and chimera activity against <sup>gst</sup>-IGD-flag, a recombinant protein substrate. A, <sup>gst</sup>-IGD-flag (17 µM) was reacted with 10 nM of each enzyme in aggrecanase reaction buffer at 37 °C for the indicated period of time. The reaction was terminated with 20 mM EDTA. The products were analyzed by SDS-PAGE (10% total acrylamide) and stained with Coomassie Brilliant Blue R-250. 8, <sup>gst</sup>-IGD-flag (17 µM) was incubated with 10 nM of each recombinant enzyme at 37 °C for 30 min. The arrows indicate the 49-kDa full-length <sup>gst</sup>-IGD-flag, the 32-kDa N terminus, and 17-kDa C terminus fragments. 32- and 17-kDa fragments are specifically generated by aggrecanase by cleavage at TEGE-ARGS. Cont, substrate control; TS4-1, ADAMTS-4-1; and TS5-5, ADAMTS-5.

Proteolytic Activity against Fibromodulin—ADAMTS-4 and -5 were previously shown to cleave fibromodulin at the Tyr<sup>48</sup>-Ala<sup>49</sup> bond (13, 14). In the case of ADAMTS-4, this activity was detected only with the Sp domain deletion mutant ADAMTS-4-2 (designated as TS4-2), but not for full-length ADAMTS-4 (21). We therefore investigated the proteolytic activity of our chimeras against fibromodulin. In agreement with previous findings (21), ADAMTS-4-2 readily hydrolyzed fibromodulin, whereas ADAMTS-4-1 had little activity (Fig. 5). Activities of chimeras were thus quantified relative to the amount of ADAMTS-4-2 cleavage in 4 h (defined as 100) (Table 1). The activity of ADAMTS-5 was 27-times greater than ADAMTS-4-1, but only ~3-times greater than ADAMTS-4-2. Addition of the TS domain of ADAMTS-5 to ADAMTS-4-1 (KF1) greatly increased the activity of ADAMTS-4, and further replacements with C-terminal domains of ADAMTS-5 (KF2 and KF3) increased activity little or only slightly. The activity of KF4 and KF5 was similar to that of ADAMTS-4-2, suggesting that non-catalytic domains of the two ADAMTSs also influence their activity for fibromodulin but differently from that on aggrecan.
Aggrecanase Activity of Full-length ADAMTS-4-1 and ADAMTS-4 Lacking the Sp Domain (TS4-2)—We previously reported that full-length ADAMTS-4 exhibits little activity against the Glu373-Ala374 bond in the IGD of the native aggrecan, whereas ADAMTS-4-2 is more effective at this site (14). However, the present study indicated that ADAMTS-4 was 18-fold more active against the IGD site than ADAMTS-4-2 and 20-fold more active against the CS-2 region (Fig. 6 and Table 2). Contrary to our previous results, our present result indicated that removal of the spacer domain is not required for full catalytic activity to cleave the Glu373-Ala374 bond. We therefore conducted further investigation to explain this discrepancy and to determine the role of the Sp domain of ADAMTS-4 in aggrecan cleavage.

Inhibition of Aggrecanase Activity of ADAMTS-4 and ADAMTS-4-2 by Heparin—A potential reason for the above discrepancy is due to contamination of our previous preparation of full-length ADAMTS-4 with heparin. As described above, we added 100 μg/ml heparin to the medium during culture to prevent recombinant ADAMTS-4-2 from adhering to the cell layer and ECM. This was not required for the preparation of ADAMTS-4-2, which does not bind to the cell surface or ECM (14). In the present study we have altered our method of purification of ADAMTS-4 by increasing the amount of NaCl in the affinity purification step and introducing an ion exchange chromatography step. We postulated that our previous method may have allowed heparin to remain bound to the purified enzyme, while our altered procedure provided heparin-free enzyme. To investigate this possibility we tested the effect of heparin on the aggrecanase activity of the present preparation of ADAMTS-4-1. ADAMTS-4-1 and ADAMTS-4-2 were incubated with various concentrations of heparin and aggrecanase activity was determined using neoepitope-specific anti-374ARGS and anti-GELE1480 antibodies. As shown in Fig. 7A, heparin inhibited ADAMTS-4 activity at the 374ARGS site much more readily than at the GELE1480 site. In contrast, heparin did not inhibit ADAMTS-4-2 activity at either site.
arin was a much less effective inhibitor of ADAMTS-4-2 at both cleavage sites (Fig. 7B). When data quantified by densitometric analysis were plotted, S-shaped inhibition curves were obtained. Based on the inhibition curve, IC50 values (concentration required for 50% inhibition) for heparin inhibition of ADAMTS-4-1 were estimated to be 4.5 μg/ml for cleavage of the 374ARGS site and 30 μg/ml for the GELE1480 site (Fig. 7A). In contrast, IC50 values for heparin inhibition of ADAMTS-4-2 at the 374ARGS and the GELE1480 sites were estimated to be more than 80 μg/ml (Fig. 7B). These results indicate that the Sp domain of ADAMTS-4-1 is essential for heparin inhibition of ADAMTS-4 activity. Thus, it is reasonable to suspect that heparin suppressed the activity of our previous full-length ADAMTS-4 against the Glu373-Ala374 bond.

To reproduce our previous data (14), ADAMTS-4 was incubated with aggrecan in the presence of 100 μg/ml heparin and its activity with ADAMTS-4-2 compared using anti-394ARGS and anti-GELE1480 antibodies (Fig. 7C). In the presence of heparin, ADAMTS-4-1 activity against the 374ARGS site was effectively suppressed, whereas high activity was still observed at the GELE1480 site. The data quantified by densitometric analysis shown in Table 2 are essentially similar to those we reported previously (14).

The Effect of Heparin on Proteolytic Activity of ADAMTS-4 against Other Protein Substrates—We also investigated the effect of heparin on ADAMTS-4-1 and ADAMTS-4-2 activity against other substrates, namely gst-IGD-flag, fibromodulin, and Cm-Tf. The time-dependent appearance of digested fragments on SDS-PAGE indicated that ADAMTS-4-1 was less active than ADAMTS-4-2 against these substrates (Fig. 8, A–C). Although the aggrecanase activity of ADAMTS-4-1 was greatly inhibited by heparin, ADAMTS-4-1 activities against these substrates were not suppressed by heparin (Fig. 8, A–C). These results indicate that, although ADAMTS-4-1 has greater aggrecanase activity, including hydrolysis of the Glu373-Ala374 bond than ADAMTS-4-2, it has much lower general proteolytic activity than ADAMTS-4-2 (Table 2). These results of the activity of the two forms of ADAMTS-4 on non-aggrecan protein substrates are similar to those previously reported (14). We therefore conclude that the Sp domain of ADAMTS-4 is essential for its effective cleavage of aggrecan both at the IGD site and in the chondroitin sulfate-rich region.

**DISCUSSION**

We have previously shown that human ADAMTS-5 is a much more active aggrecanase than human ADAMTS-4 (13, 14). To understand the different aggrecanolytic activities of the two enzymes, we investigated the function of the catalytic and non-catalytic domains in ADAMTS-4 and ADAMTS-5. As summarized in Table 1, there are a number of differences in...
Domain Functions in ADAMTS-4 and -5

![Graph](image)

**FIGURE 8.** Effect of heparin on the proteolytic activity of ADAMTS-4-1 and ADAMTS-4-2 against gst-IGD-flag, fibromodulin, and Cm-Tf. A, GST-IGD-flag (17 μM) was reacted with 10 nM of each recombinant enzyme in the presence/absence of 100 μg/ml heparin for the indicated period of time. The products were analyzed by SDS-PAGE (10% total acrylamide). B, fibromodulin (0.1 mg/ml) was reacted with 10 nM of each enzyme in the presence/absence of 100 μg/ml heparin for the indicated period of time. The products were deglycosylated using N-glycosidase F and analyzed by SDS-PAGE. C, Cm-Tf (2.5 mg/ml) was incubated with 10 nM of each enzyme in the presence/absence of 100 μg/ml heparin in aggrecanase reaction buffer at 37 °C for 4–24 h. The reaction was terminated with 20 mM EDTA. The products were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue R-250. Cont, substrate control; TS4-1, ADAMTS-4-1; and TS4-2, ADAMTS-4-2.

Domain functions between the two aggrecanases. Although we reported that the ADAMTS-5 catalytic domain is far more active than the ADAMTS-4 catalytic domain based on their activities against fibromodulin (13), it was difficult to compare the aggrecanase-specific activity of the two catalytic domains, because their catalytic domains alone were essentially inactive against aggrecan. Using chimeric enzymes, in which the catalytic domains of ADAMTS-4 and -5 were exchanged, we found that the ADAMTS-5 catalytic domain is indeed more active than the ADAMTS-4 catalytic domain by at least 4-fold in the IGD region and 2.5-fold in the CS2 region of aggrecan. This conclusion was based on comparison of KF4 with ADAMTS-4-1 and of KF3 with ADAMTS-5.

Analyses of aggrecanolytic activity of the chimeric enzymes also indicated that the non-catalytic domains of ADAMTS-5 form a more effective modifier than those of ADAMTS-4 and that they make the catalytic domains of ADAMTS-4 and -5 better aggrecanases. This was concluded from the results that the catalytic domain of ADAMTS-4 fused with the C-terminal non-catalytic domains of ADAMTS-5 (KF2 and KF3) showed significantly higher activity on aggrecan compared with ADAMTS-4-1 and that the aggrecanase activity of ADAMTS-5 was reduced when its C-terminal domains were replaced with those of ADAMTS-4 (KF4 and KF5). It was also notable that KF2 and KF3 chimeras exhibited a greater increase in cleavage at the GELE1480 site than at the IGD site. In addition, they exhibited greater activity than ADAMTS-4-1 against deglycosylated aggrecan at the GELE1480 site, although all tested enzymes were considerably less active against deglycosylated than native aggrecan (data not shown). These results suggest that the C-terminal domains of ADAMTS-5 augment activity against the CS-2 region of aggrecan by increasing the proteolytic activity of the enzyme, in addition to increasing its binding affinity for the CS chains of aggrecan. An increase in proteolytic activity is also evident from the results with fibromodulin and GST-IGD-flag (Table 1). KS chains in the IGD have been shown to influence the aggrecanolytic activity of aggrecanases (32, 33). However, the assays using the recombinant protein substrate, GST-IGD-flag lacking KS chains, showed that the chimeras had a similar increase in activity against GST-IGD-flag as against the IGD of aggrecan (Table 1). These results indicate that the involvement of KS chains in digesting the IGD region appears to be negligible.

The catalytic domains of ADAMTS-4 and -5 alone do not have any activity against Cm-Tf, but when the respective Dis domains were added to them, they exhibited 12–30% activity of full-length enzymes (13, 14). However, both KF2 and KF3 showed similar aggrecanolytic activity both at the IGD site and at the CS2 site, suggesting the Dis domain of ADAMTS-5 in the context with ADAMTS-4 catalytic domain does not affect the aggrecanase activity. In contrast, KF4, which contains the ADAMTS-5 catalytic domain and the Dis domain of ADAMTS-4, showed a 2-fold reduction in cleaving the IGD site compared with KF5, which contains both the catalytic domain and the Dis domain of ADAMTS-5. KF4 is also ~3.8-times less active than KF5 against GST-IGD-flag, but there were no significant differences in the activity at the GELE1480 site between KF4 and KF5. Taken together these results suggest that the Dis domains are essential for ADAMTS-4 and -5 to achieve basic proteolytic activity. In addition, for ADAMTS-5 to efficiently cleave the IGD domain of native aggrecan and recombinant GST-IGD-flag, the ADAMTS-5 Dis domain is necessary. Although the entire non-catalytic domains are necessary for both aggrecanases to express full aggrecanolytic activity, we hypothesize that cleavage of the IGD site is probably directly related to catalytic potency of the enzyme, whereas activity at the CS2 site is mainly dictated by the interaction between GAG chain and the non-catalytic domains located at the C-terminal of the Dis domain, most likely the Sp domain for ADAMTS-4 and the CysR domain for ADAMTS-5.

Another interesting phenomenon was that addition of the second TS domain of ADAMTS-5 to ADAMTS-4-1 (KF1) alone increased the aggrecanolytic activity of ADAMTS-4
~3-fold against the IGD region and ~4-fold against the CS-2 region of the native aggrecan. We previously showed that removal of the second TS domain from ADAMTS-5 reduced activity against the IGD site by ~50%, but it did not affect activity in the CS-2 region (13). So, a 3-fold increase in activity at the IGD site by addition of the second TS domain to ADAMTS-4-1 agrees with the previous observation, but a 4-fold increased activity at the CS2 region does not. One possible explanation for the latter phenomenon of KF1 may be that introduction of the TS domain to ADAMTS-4-1 causes conformational changes in ADAMTS-4-1 that make the C-terminal domains, particularly CysR and/or Sp domain of ADAMTS-4-1, available to interact with the GAG chains of aggrecan. Alternatively, such conformational changes may enhance general proteolytic activity. In fact, KF1 exhibited much stronger activity on fibromodulin (Table 1) and Cm-Tf (data not shown) suggesting some conformational changes take place around the Sp domain, which mimic the structure of ADAMTS-4-2 with an increased activity against fibromodulin and Cm-Tf. These observations suggest that the C-terminal domain arrangement of ADAMTSs is dynamic, but the proof of this concept awaits resolution of the three-dimensional structures of these proteinases.

In addition to the chimera study, we re-investigated the role of Sp domain in the proteolytic activity of ADAMTS-4. We (13, 14) and others (25, 34) have previously reported that ADAMTS-4-1 exhibits little activity against the Glu373-Ala374 bond in the IGD of aggrecan, whereas ADAMTS-4-2 is more effective at this site. On the other hand, Hashimoto et al. (35) reported that full-length ADAMTS-4 was as active as a form of the enzyme lacking the spacer domain in cleaving the Glu373-Ala374 bond, suggesting that removal of the spacer domain is not required for full catalytic activity. Contrary to our previous report (14), our new preparation of ADAMTS-4-1 had potent activity against the Glu373-Ala374 bond in the IGD of aggrecan, and ADAMTS-4-2 had only 4–5% of the ADAMTS-4-1 activity at both IGD and CS2 sites. This discrepancy is most likely due to heparin contamination of our previous preparation of ADAMTS-4-1, because heparin inhibited ADAMTS-4-1 but not ADAMTS-4-2 activity against the IGD of aggrecan. We were also able to replicate our previous data by adding heparin back to the enzyme preparations. Based on these studies we concluded that full-length ADAMTS-4 is much more active on aggrecan at both the IGD and CS2 regions of aggrecan than ADAMTS-4-2, but full-length ADAMTS-4 is still a 22- to 33-fold weaker aggrecanase than full-length ADAMTS-5. ADAMTS-4-2 has greater general proteolytic activity against Cm-Tf, fibromodulin, and gst-IGD-flag than full-length ADAMTS-4-1. ADAMTS-4-2 also cleaves biglycan and decorin (14, 36). Since the Sp domain of ADAMTS-4-1 interacts with the cell surface and pericellular sulfated proteoglycans, it is likely that the site of action of full-length ADAMTS-4 is around the chondrocytes. In this location, it is likely that the activity on the IGD site may be reduced due to interaction with and inhibition by sulfated proteoglycans of the pericellular ECM. Processing of the Sp domain of ADAMTS-4 by MT4-MMP (26) releases ADAMTS-4-1 from the matrix and greatly reduces its aggrecanolytic activity, but the enzyme gains the ability to cleave substrates other than aggrecan and is less sensitive to inhibition by sulfated proteoglycans. A similar scenario may apply to ADAMTS-5, as it also binds to the cell surface and ECM primarily through its CysR domain (13). However, the inhibitory effect of heparin on full-length ADAMTS-5 activity at both the IGD and the CS2 sites was much weaker (IC50 = 60–80 μg/ml) compared with heparin inhibition of ADAMTS-4-1 activity at the IGD site. So, ADAMTS-5 bound to the matrix is far more active than ADAMTA-4. ADAMTS-5 processed at or before the CysR domain would lose 99% of its aggrecanolytic activity but would retain ~25% of its general proteolytic activity (13). Cleavage of non-aggrecan molecules such as biglycan and fibromodulin in the inter-territorial region of the cartilage will significantly affect the homeostasis of the cartilage.

This and our previous studies indicate that the C-terminal domains of ADAMTS-4 and -5 govern the specificity of the enzymes by modulating substrate binding. Agents able to block interaction of the C-terminal domains with endogenous substrates thus have the potential to act as substrate-specific exosite inhibitors of the aggrecanases.

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