ADAMTS-4 (a disintegrin and metalloprotease with thrombospondin motifs) is a multidomain metalloprotease belonging to the repysolin family. The enzyme cleaves aggrecan core protein at several sites. Here we report that the non-catalytic ancillary domains of the enzyme play a major role in regulating aggrecanase activity, with the C-terminal spacer domain masking the general proteolytic activity. Expressing a series of domain deletion mutants in mammalian cells and examining their aggrecan-degrading and general proteolytic activities, we found that full-length ADAMTS-4 of 70 kDa was the most effective aggrecanase, but it exhibited little activity against the Glu[1480]-Ala[1481] bond, the site originally characterized as a signature of aggrecanase activity. Little activity was detected against reduced and carboxymethylated transferrin (Cm-Tf), a general protease substrate. However, it readily cleaved the Glu[1480]-Gly[1481] bond in the chondroitin sulfate-rich region of aggrecan. Of the constructed mutants, the C-terminal spacer domain deletion mutant more effectively hydrolyzed both the Glu[1480]-Ala[1481] and Glu[1480]-Gly[1481] bonds. It also revealed new activities against Cm-Tf, fibromodulin, and decorin. Further deletion of the cysteine-rich domain reduced the aggrecanase activity by 80% but did not alter the activity against Cm-Tf or fibromodulin. Further removal of the thrombospondin type I domain drastically reduced all tested proteolytic activities, and very limited enzymatic activity was detected with the catalytic domain. Full-length ADAMTS-4 binds to pericellular and extracellular matrix, but deletion of the spacer domain releases the enzyme. ADAMTS-4 lacking the spacer domain has promiscuous substrate specificity considerably different from that previously reported for aggrecan core protein. Finding of ADAMTS-4 in the interleukin-1α-treated porcine articular cartilage primarily as a 46-kDa form suggests that it exhibits a broader substrate spectrum in the tissue than originally considered.

ADAMTS-4 is a zinc metalloprotease that belongs to the repysolin subfamily of metalloendoproteinase M12 family as the metalloprotease domain is related to snake venom metalloproteases, reprolysins (1). It has also been designated as “aggrecanase 1” based on its ability to cleave the Glu[1480]-Ala[1481] bond located within the interglobular domain (IGD) of the aggrecan core protein (2), an original characteristic property proposed for aggrecanases (3). The enzyme is considered to participate in early stages of cartilage destruction in rheumatoid arthritis and in osteoarthritis as well as normal turnover of aggrecan (4, 5).

Bovine and porcine articular cartilage (6–8), human chondrocytes (9), and bovine chondrocytes (6) treated with IL-1 all have increased the levels of ADAMTS-4 mRNA. Human synovial fibroblasts also increase both mRNA and protein levels of ADAMTS-4 when treated with transforming growth factor β (10). The enzyme is a multidomain metalloproteinase, consisting of a large propeptide, a catalytic metalloproteinase domain, a disintegrin (Dis), a thrombospondin type I (TS), a cysteine-rich (CysR), and a spacer (Sp) domain (2). Besides the TEGE[1771]–ARGS bond, recombinant ADAMTS-4 expressed in insect cells cleaves the core protein at GELE[1480]–GRGT, KEEE[1696]–GLOS, TQAE[1571]–AGEG, and VSQE[1871]–LGQR sites (indicates the bond cleaved) in the chondroitin sulfate-rich CS-2 region of bovine aggrecan core protein (11). These sites are more readily cleaved than the originally characterized cleavage Glu[1480]-Ala[1481] bond (11). On the other hand, the aggrecanase activity was not detected when the catalytic domain of ADAMTS-4 was expressed, although it retained the activity against a 41-residue synthetic peptide encompassing the Glu[1480]-Ala[1481] bond (12, 13). It has also been reported that ADAMTS-4 is unable to cleave the core protein when aggrecan was deglycosylated by chondroitinase ABC and keratanase (12). Interaction of the TS domain of the enzyme and anionic polysaccharide chains of aggrecan is considered to be important for the expression of aggrecanase activity, because a peptide harboring a potential heparan sulfate binding motif W(S/G)WX (14) inhibited the aggrecanase activity with an IC₅₀ value in the low micromolar range (12). More recently,
however, Gao et al. (15) have reported that the full-length ADAMTS-4 expressed in mammalian cells exhibits little aggrecanase activity as determined by the detection of fragments with the newly generated N-terminal Δ373ARGSVΔ378 sequence using a neoepitope antibody, but that this activity was greatly increased when the full-length ADAMTS-4 was processed to 60- and 50-kDa forms by removing the C-terminal part of the enzyme. Similar C-terminal processing was also reported that the recombinant ADAMTS-4 expressed in Chinese hamster ovary/A2 cells was autolytically processed to 53 and 40 kDa by cleaving Lys694–Phe695 and Thr581–Phe582 bonds, and both forms cleave aggrecan at the Glu373–Ala374 bond (16). Thus, it is not clear which domains of the enzyme play a key role in aggrecanase activity.

The aim of this study is to investigate the role of the non-catalytic ancillary domains of ADAMTS-4 in aggrecan degradation. For this purpose, we have systematically deleted the ancillary domains from the C terminus and measured the aggrecanase activity of the truncated forms using four different assay methods: i) an aggrecan-polyacrylamide beads assay (17); ii) detection of core protein fragments using the antibody 2-B-6 that recognizes the chondroitinase-generated chondroitin-4-sulfate “stubs” for general aggrecan degradation products (18); (iii) detecting the cleavage at the Glu373–Ala374 bond by the Glu373–Ala374 antibody (19) and (iv) at the Glu1420–Gly1421 bond in the CS-2 region using specific neoepitope antibodies. We have also examined other protein substrates, including reduced, carboxymethylated transferrin (CM-Tf). The results show that considerable changes occur in substrate specificity upon deletion of each domain. Full-length ADAMTS-4 binds to the extracellular matrix (ECM) and the cell surface, but the deletion of the C-terminal Sp domain releases the enzyme from these sites. Direct extraction of ADAMTS-4 from porcine articular cartilage treated with interleukin-1α (IL-1α) indicated that the majority of the enzyme in such tissues lacks a portion of the C-terminal domains. Our studies suggest that ADAMTS-4 in the tissue may have a broader substrate spectrum than has previously been considered and trafficking of the enzyme is also regulated in the extracellular space by C-terminal processing.

**EXPERIMENTAL PROCEDURES**

**Materials**—pCEP4 plasmid vector, pMT/V/HisA vector, and 293-EBA cells were from Invitrogen (Groningen, The Netherlands). Restriction enzymes, T4 DNA ligase, and peptide N-glycosidase F (PNGase F) were from New England Biolabs (Hitchin, UK). Pfu Turbo DNA polymerase was from Stratagene Europe (Amsterdam, The Netherlands). HTB-94 human chondrosarcoma cell line was from American Type Cell Collection. FuGENE 6 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). Precision protein standard was from the Bio-Rad Laboratory Ltd. (Hemel Hempstead, UK). Recombinant interleukin-1α (IL-1α) was a gift from Prof. J. Saklatvala (Imperial College London, UK). Anti-FLAG M2 antibody (mouse monoclonal antibody), anti-FLAG M2-agarose, decorin, biglycan, and fibromodulin were from Sigma (Dorset, UK). Goat serum was from DAKO (Ely, UK). The Alexa-488-conjugated goat anti-(mouse IgG) IgG, Alexa-568-conjugated goat anti-(rabbit IgG) IgG, and Alexa-647-labeled phalloidin were from Molecular Probes (Leiden, The Netherlands). Chondroitin ABC and keratanase were from Seikagaku Kogyo (Tokyo, Japan). Sephacryl S-200, and N-hydroxysuccinimide-activated Sepharose were from Amersham Biosciences (Little Chalfont, UK). Cm-Tf was prepared as described previously (20). Aggrecan monomers were purified from bovine nasal cartilage under dissociative conditions according to Hancock and Sajdera (21). Human recombinant TIMP-1 and TIMP-2 were expressed in mammalian cells and purified as described previously (22, 23). The N-terminal domain of human TIMP-3 with C-terminal His tag (N-TIMP-3-His) was expressed in Escherichia coli, folded in vitro and purified (24). N-TIMP-3-Sepharose was prepared by coupling 1 mg of N-TIMP-3 to 2 ml of NHS-activated Sepharose according to the manufacturer’s instruction. Ni-NTA-agarose was from Qiagen Ltd. (Crawley, UK).

**Anti-ADAMTS-4 Antibody and Neoepitope Antibodies against Aggrecan**—The antisera against human ADAMTS-4 was raised in a rabbit using the purified recombinant catalytic domain of the enzyme. The antibody was rendered specific to ADAMTS-4 after purification by an affinity chromatography using Sepharose coupled with the catalytic domain of ADAMTS-4. The antibody that recognizes the aggrecanase-cleaved C-terminal neoepitope –TAGELE1460 was raised in a rabbit using the peptide CGGTAGLE linked to keyhole limpet hemocyanin. Monoclonal antibodies, BC-3 that recognizes the N-terminal neoepitope Δ373–Δ378 of aggrecan core protein and 2-B-6 that recognizes the chondroitinase-generated chondroitin-4-sulfate “stubs” of aggrecan core protein were prepared and characterized as described by Hughes et al. (19) and Caterson et al. (18), respectively.

**Construction of cDNA Coding for ADAMTS-4 and the C-terminal Deletion Mutants**—cDNA encoding human ADAMTS-4 was obtained by reverse transcription-polymerase chain reaction using the total RNA extracted from human primary chondrocytes. The PCR was carried out for 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 60 °C), and extension (1 min at 72 °C) using Pfu Turbo DNA polymerase with specific primers, derived from human ADAMTS-4 DNA sequence (NCBI accession number: NM050199). The two primers used were: 5′-ATGTGCGAGAAGGCTGATACCC-3′ and 3′- reverse primer GGTATTTTCCTGCCCG-5′. A PCR fragment (1235 bp) was ligated into pUC19 plasmid vector (pUC19-ADAMTS-4), and the nucleotide sequence determined and confirmed as human ADAMTS-4. To construct the vector expressing the full-length ADAMTS-4 (TS4-1), PCR was first performed with the forward primer, 5′-GGAATTCAGATGGATCCGCGCGCTG-3′ (ADAMTS-4/FW/EcoRI containing EcoRI site (underlined), Kozak consensus sequence (in italic) (25) and ADAMTS-4 N-terminal sequence, and the reverse primer, 5′-GTCACTGCTATTTTATATACTTCTGGCCCGCCAGGG-3′ (TS4-IR/FLAG) containing the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys-stop) (in italic) and the C-terminal sequence of ADAMTS-4 using the pUC19-ADAMTS-4 vector as a template. The second PCR was carried out using the first PCR product as a template and the same 5′ forward primer as the first PCR (ADAMTS-4/FW/EcoRI) and the 3′ reverse primer 5′-TAGAATTCGATTAATCTGTCATGTCATTTAAT-3′ (FLAG/RV/XhoI) carrying XhoI site (underlined), Kozak consensus sequence (in italic) (25) and ADAMTS-4 N-terminal sequence, and the reverse primer, 5′-GTCACTGCTATTTTATATACTTCTGGCCCGCCAGGG-3′ (TS4-IR/FLAG) containing the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys-stop) (in italic) and the C-terminal sequence of ADAMTS-4. The PCR products were sequenced and confirmed to be the pCEP4 vector as described above. The inactive ADAMTS-4(E362A) mutant was constructed by PCR using “megaprimer” method (26) with primers 5′-GTCATGCGGATCCCCGATGACCTGTAGTGT-3′ (forward) and 5′-ACCAGAGGCTGATGAGCACTGAGTGGTA-3′ (reverse), where the original codon GAA encoding Glu was mutated to GCC encoding Ala (underlined).

**Expression and Purification of the Recombinant ADAMTS-4 and Its Variants**—The pCEP4 vector harboring ADAMTS-4 or its variants was transfected into 293-EBA cells by lipofection with FuGENE6. The stably transfected cells were selected for hygromycin (300 μg/ml) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 units/ml). To obtain the recombinant protein, the culture media were replaced with DMEM containing 0.2% lactalbumin hydrolysate, penicillin, and streptomycin, and the conditioned media were harvested once a week. Full-length ADAMTS-4 (TS4-1) secreted by cells bound to the cell surface and ECM, and little was detected in the medium. Thus, 100 μg/ml heparin was added during the culture, which released TS4-1 into the medium. The collected conditioned media (1 liter) were centrifuged to remove cell debris and applied to a column of anti-FLAG M2-agarose (2 ml) for purification. The material bound to the column was eluted with 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM CaCl2, and 0.02% NaN3. The eluted recombinant ADAMTS-4 was further purified by gel filtration on S-200 Sephadryl equilibrated with 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 10 mM CaCl2, and 0.02% NaN3 (TNC buffer).
About 80% of aggrecanase activity in the conditioned medium of TS4-1 did not bind to anti-FLAG M2 agarose. To purify TS4-1 in the flow-through fraction, it was applied to a column of N-TIMP-3-Sepharose. The bound material was eluted with 6 M urea in 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl. The eluate was then dialyzed extensively against TNC buffer. The domain deletion variants were secreted into the medium. They were purified by anti-FLAG affinity chromatography and gel filtration as described above. The concentration of active recombinant ADAMTS-4 and variants were determined by titration with the known concentrations of N-TIMP-3.

SDS-PAGE and Western Blotting—SDS-PAGE was carried out according to Bury (27), and proteins were stained by silver (28). Precision protein standards were used as molecular weight markers. For Western blotting analyses, proteins separated by SDS-PAGE were electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked by 5% dry milk solution and reacted with the primary antibody anti-FLAG M2 antibody (1:2000 dilution), anti-ADAMTS-4 catalytic domain antibody (1:1000 dilution), BC-3 neoeptope antibody (1:200 dilution), anti-GELE antibody (1:2000 dilution), or 2-B-6 antibody (1:1000 dilution). The antigen-antibody complexes were then reacted with the second antibody conjugated with alkaline phosphatase, and the protein bands were visualized using Western Blue stabilized substrate.

Immunolocalization of ADAMTS-4—Human chondrosarcoma HT-115 cells were seeded and grown on gelatin-coated glass coverslips and transfected with the pCEP4 expression vector containing ADAMTS-4 cDNA or its variants by lipofection with FuGENE6. In the following day the cells were washed once with serum-free DMEM and incubated in serum-free DMEM for 48 h. The cell layers were then reacted with phosphatase-substituted saline (PBS), fixed using 4% (v/v) paraformaldehyde in PBS for 7 min, washed with PBS four times and incubated in blocking solution (3% (v/v) bovine albumin and 5% (v/v) goat serum in PBS) for 1 h at room temperature. The cell layers were then reacted with the primary antibodies, the mouse anti-FLAG M2 antibody (1:2000 dilution), and rabbit anti-catalytic domain of human ADAMTS-4) IgG (1:500 dilution) in the blocking solution for 2 h at room temperature. The specimens were then washed twice with PBS and incubated with fluorescence-labeled secondary antibodies (Alexa-488-conjugated goat anti-mouse IgG) IgG (1:500 dilution) and Alexa-568-conjugated goat anti-rabbit IgG) IgG (1:500 dilution) in blocking solution for 1 h at room temperature. The specimens were then washed twice with PBS and incubated with fluorescein isothiocyanate (FITC) and Texas red-X (TRX) labeled neoepitope antibodies (Alexa-488-conjugated goat anti-mouse IgG) (1:500 dilution) and Alexa-568-conjugated goat anti-rabbit IgG) (1:500 dilution) in blocking solution for 1 h at room temperature. The specimens were then washed twice with PBS and incubated with fluorescence-labeled secondary antibodies (Alexa-488-conjugated goat anti-mouse IgG) (1:500 dilution) and Alexa-568-conjugated goat anti-rabbit IgG) (1:500 dilution) in blocking solution for 1 h at room temperature. The specimens were then washed twice with PBS and incubated with Texas red-X (TRX) labeled neoepitope antibodies (Alexa-488-conjugated goat anti-mouse IgG) and Alexa-568-conjugated goat anti-rabbit IgG) in blocking solution for 1 h at room temperature.

Expression of the Recombinant ADAMTS-4 and C-terminal Domain Deletion Variants—Full-length mature form of ADAMTS-4 and the C-terminal domain deletion enzymes were purified from conditioned medium by anti-FLAG affinity chromatography and gel filtration on Sepharcll S-200. All preparations were homogenous on SDS-PAGE when stained with silver (Fig. 2A). Purified materials were also detected by Western blotting analysis with an anti-FLAG antibody (Fig. 2B) and anti-catalytic domain antibody (Fig. 2C). Approximately 50 μg of the 70-kDa TS4-1 (full-length), 520 μg of the 53-kDa TS4-2 (Sp deletion), 620 μg of the 40-kDa TS4-3 (Sp and CysR deletion), 1.8 mg of the 35-kDa TS4-4 (Sp, CysR, and TS deletion), and 2.6 mg of the 27-kDa TS4-5 (Sp, CysR, and TS deletion) were loaded onto each lane of a 10% polyacrylamide gel for SDS-PAGE and Western blotting.
1.2 mg of the 26-kDa TS4-5 (the catalytic domain), and 110 μg of the 70-kDa TS4-1(E362A) were purified from 1 liter of conditioned media. The N-terminal sequence analysis of the isolated ADAMTS-4 was Phe-Ala-Ser-Leu-Ser, indicating that the propeptide was processed intracellularly and mature forms were secreted from the cell. Full-length TS4-1 was largely found on the cell surface or bound to the ECM (see below), but the addition of heparin to the culture released the majority of TS4-1 into the medium. However, about 80% of the aggrecanase activity in the conditioned medium of TS4-1 did not bind to the anti-FLAG column, indicating a loss of the C-terminal FLAG during culture. When the unbound fraction was applied to a column of N-TIMP-3-Sepharose and eluted with 6M urea in 50 mM Tris-HCl (pH 8.0) containing 500 mM NaCl, two major species of ADAMTS-4 of 68 kDa and 53 kDa and a minor fragment of 58 kDa were detected following Western blotting analysis with the anti-catalytic domain antibody (Fig. 2D).

Similarly, an inactive ADAMTS-4(E362A) mutant (TS4-1(E362A)) was also found to be processed (data not shown), indicating that the post-secretory processing of ADAMTS-4 is not due to autoproteolysis. The majority of the other domain deletion mutants bound to the anti-FLAG column.

The full-length ADAMTS-4 Binds to the Cell Surface and ECM—Transfection of chondrosarcoma cell line HTB-94 with the full-length TS4-1 construct showed little enzyme activity in the medium. Immunolocalization of TS4-1 in HTB-94 cells with anti-catalytic domain and anti-FLAG antibodies indicated that TS4-1 was associated with the cell surface and ECM (Fig. 3). The addition of heparin to the culture released most of TS4-1 molecules, suggesting that it may bind to negatively charged cell surface and matrix proteoglycans. On the other hand, the Sp domain deletion mutant TS4-2 did not bind to the ECM or cell surface (Fig. 3) and was recovered from the medium. Other domain deletion mutants were also found in the medium, indicating that the C-terminal Sp domain is responsible for cell surface and ECM binding of ADAMTS-4.

Aggrecanase Activity of ADAMTS-4 and Domain Deletion Mutants—Aggrecan-degrading activities of the full-length and domain deletion mutants were first measured by the aggrecan-polyacrylamide bead assay. The results are summarized in Table I. TS4-1 was most active with a specific activity of 39.8 ± 1.0 units/nmol. TS4-2 exhibited a 28% loss of aggrecanase activity (28.8 ± 2.3 unit/nmol), and further deletion of the CysR domain (TS4-3) resulted in an 81% loss of aggrecanase activity. Only about 1% of the activity was detected with TS4-4, and no aggrecanase activity was detected with the catalytic domain (TS4-5) alone. The ADAMTS-4(E362A) mutant exhibited no enzymatic activity. These results indicate that the Sp domain and the CysR domain play major roles in the aggrecanase activity of ADAMTS-4.

The aggrecan-degrading activity of these variants was also investigated using neoepitope antibodies recognizing either the N-terminal 574-ARGSV-- neoepitope representing cleavage in the IGD, or the C-terminal --GELE469 neoepitope representing cleavage in the CS-2 region (Fig. 4B). The monoclonal antibody 2-B-6 was used to monitor the overall degradation pattern of
Altered Proteolytic Activities of ADAMTS-4

TABLE I

Aggrecan-degrading activity of ADAMTS-4 and domain deletion mutants measured by the aggrecan-polyacrylamide-bead assay

| Enzyme Mutant | Aggrecan-degrading activity units/nmol protein |
|---------------|----------------------------------------------|
| TS4-1 Wild-type | 39.8 ± 1.0 |
| TS4-2 ΔSp | 28.7 ± 2.3 |
| TS4-3 ΔCyRS and Sp | 7.8 ± 0.2 |
| TS4-4 ΔTS, CyRS and Sp | 0.4 ± 0.0 |
| TS4-5 ΔDis, TS, CyRS and Sp | ND* |
| TS4-1(E362A) | ND* |
| MMP-3(ΔC) | 13.8 ± 0.2 |

*ND, not detected.

core protein was cleaved by TS4-2, TS4-3, TS4-4, and the catalytic domain (TS4-5). The Glu1450-Gly1451 bond of the deglycosylated core protein was cleaved by TS4-2, TS4-3, and TS4-4, but not by TS4-1 or TS4-5. Taken together, these results indicate that interaction between the C-terminal Sp domain and the GAG chains of aggrecan is essential for TS4-1 to cleave the CS-2 region including the Glu1450-Gly1451 bond, because the enzyme is not active when aggrecan was deglycosylated. Once the Sp domain is truncated, the enzyme could act on the deglycosylated aggrecans, although the activity was weaker compared with that on the native aggrecan. In addition, the cleavage patterns generated by TS4-2 and TS4-3 of the native and those of the deglycosylated aggrecan core protein were sufficiently different to suggest that the substrate specificity of TS4-2 and TS4-3 is altered by negatively charged polysaccharide chains.

Proteolytic Activity of ADAMTS-4 against Other Protein Substrates—Because the deglycosylated aggrecan was cleaved by some of the domain-deletion mutants, we examined the five variants for their proteolytic activities against various other proteins. None of the enzyme constructs could digest gelatin, fibronectin, or casein (data not shown). However, TS4-2 and TS4-3 similarly digested Cm-Tf into numerous fragments to a similar extent. A considerably weaker activity was detected with TS4-4, and little activity was detected with TS4-1 and TS4-5 (Fig. 6A). The digestion of Cm-Tf by ADAMTS-4 variants was completely inhibited by 20 nM TIMP-3 but not by TIMP-1 or TIMP-2 at 100 nM (Fig. 6B), indicating that the proteolytic activity is solely derived from ADAMTS-4 after C-terminal truncation. This led us to postulate that processed forms of ADAMTS-4 may have proteolytic activity against cartilage matrix components other than aggrecan. We therefore tested biglycan, decorin, and fibromodulin as potential substrates. As shown in Fig. 7, TS4-2 digested decorin and fibromodulin but not biglycan. Among ADAMTS-4 variants, TS4-2 and TS4-3 digested fibromodulin most effectively, but only a weak activity was seen with TS4-1 (Fig. 7D).

Analysis of ADAMTS-4 Cleavage Sites in Cm-Tf and Fibromodulin Shows Novel Substrate Specificity—To investigate the substrate specificity of the C-terminal Sp domain-deleted ADAMTS-4, Cm-Tf was digested by TS4-2 for various times, and the products were separated by SDS-PAGE and subjected to N-terminal sequence analyses (Fig. 8A). Several fragments showed double or triple sequences, and in those cases the recovery of phenylthiohydantoin amino acids were essentially equal. The time course of digestion indicated that the major products were bands 4, 24, 25, 39, 43, 50, 55, and 63 (the numbers infer molecular masses). Aligning the fragments and the time-dependent appearance of fragments on SDS-PAGE...
indicating faster and slower cleavage (Fig. 8B). Because transferrin consists of two homologous domains (31), alignment of the two corresponding sequences has provided additional insights into substrate specificity for cleavage (Fig. 8B). It is generally considered that ADAMTS-4 cleaves on the carboxyl side of glutamic acid residues (Glu-X bond), but cleavage sites identified in Cm-Tf were after Met, Ser, Leu, Gln, His, Phe, Gly as well as side chains with a carboxyl group such as Cm-Cys and Asp. The N-terminal sequence of the 29-kDa fragment of fibromodulin was Ala45-Tyr46-Gly47-Ser48-Pro49, indicating that the Tyr 44-Ala45 bond was cleaved by the C-terminal domain truncated ADAMTS-4.

**Identification of ADAMTS-4 in Porcine Articular Cartilage Treated with IL-1β** —To identify the molecular species of ADAMTS-4 in cartilage, porcine articular cartilage was treated with IL-1β/H9251 for 3 days, and the cartilage proteins were extracted with 4 M guanidine hydrochloride. The extract was incubated with TIMP-1 and N-TIMP-3-His during the renaturation process, and N-TIMP-3-bound materials were subjected to Western blotting analysis with the antibody raised against the catalytic domain of ADAMTS-4 (Fig. 9). The cartilage without IL-1β/H9251 exhibited small amounts of 40- and 37-kDa species. IL-1β/H9251-treated cartilage contained an increased amount of a 46-kDa species. The full-length 70-kDa ADAMTS-4 was not detected under these experimental conditions, suggesting that the enzyme is processed in the tissue, lacking the Sp domain and a part of the CysR domain.

**DISCUSSION**

**The Role of Non-catalytic Domains of ADAMTS-4 in Aggrecan Digestion** —ADAMTS-4 has been considered to act specifically on aggregating proteoglycans such as aggrecan (2), brevican (32), and versican (33), but not other proteins (34). Tortorella et al. (11) reported that the recombinant enzyme

![Fig. 4. Detection of aggrecanase activities of ADAMTS-4 and domain-deletion mutants by neoepitope antibodies.](http://www.jbc.org/content/early/2007/07/16/jbc.M701418200F0004.large.jpg)

![Fig. 5. Effect of GAG chains on the aggrecanase activity of ADAMTS-4.](http://www.jbc.org/content/early/2007/07/16/jbc.M701418200F0005.large.jpg)
cleaves the native aggrecan core protein at the Glu373-Ala374 bond, although it cleaves the chondroitin sulfate-rich region more effectively. More recent studies of Gao et al. (15), however, described that full-length ADAMTS-4 had little activity against the Glu373-Ala374 bond, whereas the C-terminal processing of the enzyme to 60 and 50 kDa by MMPs greatly increased the activity against this site. Our systematic mutagenesis studies have elucidated that each domain significantly influences the substrate specificity of the enzyme. Full-length ADAMTS-4 was most effective in digesting aggrecan, but it had an extremely weak activity in cleaving the “classic” aggrecanase site, the Glu373-Ala374 bond. Among the five variants tested, the most active species for this bond was TS4-2, which lacks the C-terminal Sp domain. TS4-3, which lacks both the Sp and CysR domains, retained about 30–40% of the activity. These results essentially agree with the observations by Gao et al. (15). On the other hand, full-length ADAMTS-4 has the most effective aggrecan-degrading activity when measured by the bead assay. This activity is most likely due to the cleavage of the chondroitin sulfate-rich region of aggrecan as judged by its ability to cleave the Glu1480-Gly1481 bond. Further deletion of the CysR domain drastically reduced aggrecan-degrading activity and the cleavage of the Glu1480-Gly1481 bond. These results suggest that the CysR domain has a major contribution in cleaving the chondroitin sulfate-rich region of aggrecan. Deletion of the Sp domain revealed a novel proteolytic activity on Cm-Tf. This activity coincides with a large increase in cleaving the Glu373-Ala374 bond in the aggrecan interglobular domain, where limited carbohydrates are present and with increased degradation of the core protein of deglycosylated aggrecan. For these proteolytic activities, the Sp domain acts as an intramolecular suppressor. Our study also indicates that the Dis domain plays a essential role in activity against Cm-Tf, because TS4-5 exhibits little activity on Cm-Tf. TS4-3 has significantly higher activity against Cm-Tf than TS4-4, suggesting that both Dis and TS domains optimize the expression of general proteolytic activities. Although the Sp domain inhibits the activities on Cm-Tf and the Glu373-Ala374 bond of aggrecan, it does not inhibit the aggrecan-degrading activity at the chondroitin sulfate-rich region. One possible explanation for this differential activity may be that the Sp domain interacts with the chondroitin sulfate chains as suggested from the immunolocalization studies of TS4-1 (see Fig. 3). Such interaction may suppress the inhibitory nature of the Sp domain of ADAMTS-4. Although the Sp domain inhibits the activities on Cm-Tf and the Glu373-Ala374 bond of aggrecan, it does not inhibit the aggrecan-degrading activity at the chondroitin sulfate-rich region. One possible explanation for this differential activity may be that the Sp domain interacts with the chondroitin sulfate chains as suggested from the immunolocalization studies of TS4-1 (see Fig. 3). Such interaction may suppress the inhibitory nature of the Sp domain of ADAMTS-4. However, TS4-1 does not express Cm-Tf activity in the presence of chondroitin sulfate chains, indicating that further investigation is necessary to understand the exact mechanism by which the Sp domain regulates the hydrolysis of aggrecan core protein and Cm-Tf.

Another unique feature of ADAMTS-4 is that the catalytic domain by itself has a very limited proteolytic activity. Neither aggrecanolytic activity nor Cm-Tf-digesting activity was detected with TS4-5, although deglycosylated aggrecan core pro-
FIG. 7. Digestion of small leucine-rich proteoglycans. Bovine biglycan (BGN) (0.5 mg/ml), bovine decorin (DCN) (0.5 mg/ml), and bovine fibromodulin (FMOD) (0.1 mg/ml) were incubated with 50 nM TS4-2 in TNC buffer at 37 °C for the indicated periods of time and the reactions were stopped by 10 mM EDTA. One-half of the samples was directly subjected to SDS-PAGE and staining with 0.2% Toluidine Blue O (left panels). The remainder was deglycosylated by chondroitinase ABC for biglycan and decorin or by PNGase for fibromodulin and subjected to SDS-PAGE and proteins were stained with Coomassie Brilliant Blue R-250 (right panels). A, biglycan; B, decorin; C, fibromodulin; D, fibromodulin digested by ADAMTS-4 variants (10 nM) for indicated time periods.
tein was very slowly cleaved into several fragments. The catalytic domain also cleaved the bait region of \( \text{H\textasciitilde}9251 \) indicating that it is proteolytically active. Such poor proteolytic property of the catalytic domain of ADAMTS-4 appears to be unusual, because most proteinase domains are usually active by themselves on certain substrates while ancillary domains participate in regulation of the activity and specificity of the proteolytic activity.

\[ \text{3 M. Kashiwagi, L. Sottrup-Jensen, and H. Nagase, unpublished results.} \]

FIG. 8. Sites cleaved in Cm-Tf by TS4-2. A. Cm-Tf (2.5 mg/ml) was incubated with 50 nM TS4-2 for the indicated periods of time. The reaction was terminated by 10 mM EDTA, and the fragments were separated by SDS-PAGE (15% total acrylamide) and subjected to N-terminal amino acid sequence analysis as described under “Experimental Procedures.” The N-terminal sequence of each fragment is indicated together with its size in kDa before the sequence. “V” indicates the N-terminal Val of human transferrin. B. Alignment of sequences around the TS4-2 cleavage sites and the corresponding homologous sequences in the other domain of Cm-Tf. According to the cleavage pattern shown in A, the cleavage sites were categorized into “faster cleavage” and “slower cleavage” sites. The actual cleavage sites are indicated by bold arrows for faster cleavage, and thin arrows for slower cleavage. The underlined “C” is carboxymethylated cysteine.
enzyme. The catalytic domain of matrix metalloproteinase 1 (MMP-1) is proteolytically active and digests casein, gelatin, and synthetic peptide substrates as effectively as the full-length MMP-1, but it fails to cleave interstitial fibrillar collagens without the hemopexin domain (35, 36). Only the full-length MMP-1 can cleave collagens. Differently processed forms of plasmin also exhibit altered activities on fibrinogen and fibrin, but microplasmin that lacks five kringle domains and synthetic peptide substrates as effectively as the full-length MMP-1, but it fails to cleave interstitial fibrillar collagens without the hemopexin domain (35, 36). Only the full-length MMP-1 can cleave collagens. Differently processed forms of plasmin also exhibit altered activities on fibrinogen and fibrin, but microplasmin that lacks five kringle domains retains its activity on both substrates, although reduced (37). It would be interesting to investigate whether the poor proteolytic activity of the catalytic domain is common to ADAMTS and ADAM metalloproteinases.

**Substrate Specificity of ADAMTS-4**—The substrate specificity of ADAMTS-4 was first characterized by determining the sites cleaved in bovine aggrecan at Glu373-Ala374, Glu1480-Gly1481, Glu1666-Gly1667, Glu1771-Ala1772, and Glu1805-Leu1806 bonds (11) and at the Glu295-Ser3096 bond in brevican (32) and the Glu441-Ala442 bond in versican (33). These results suggest that the presence of a glutamic acid residue at the P1 position is one of the key determinants of substrate specificity. This study has, however, revealed that the enzyme has broader substrate specificity if the Sp domain is removed. N-terminal sequencing analysis demonstrated that among eight most susceptible sites of Cm-Tf only one site contains C and P residues at the P1 subsite, but the residues at the P1 position of other cleavage sites are promiscuous, including Met, Ser, Leu, Gly, and Asp. The residues at the P1' position found in aggrecan core protein are Gly, Ala, and Leu (11), but those in Cm-Tf are also promiscuous, including Leu, Tyr, Phe, Asp, and Gly. One of the advantages of using Cm-Tf for investigation of substrate specificity is that human transferrin consists of duplicated homologous domains. Alignment of the cleaved sequences and their counterpart sequences thus provides interesting insights into the sequence specificity of the enzyme. An example is the pair of “a-1” and “a-2” in Fig. 8B, where the major substitutions are found in P2', P4', and P6' positions, but the sequence of P6'-P10' in peptide a-1 is identical to that of the P5'-P9' in peptide a-2. The latter region was cleaved more slowly by TS4-2 or TS4-3. This pair will be a good model to further investigate the substrate requirements of ADAMTS-4. Other interesting results are the triplet cleavage at the SLDG (408–411) site in peptide “c-1” and double cleavage in the sequence encompassing the “b-1” and “h-1” region. These cleavages are either due to independent endopeptidase actions, or due to exopeptidase activities of TS4-2 after cleavage of one of these sites. If they were all due to endopeptidase activity, this would suggest that the sequence requirement for these cleavages are not strict and the linear sequences around the cleavage sites may not be the determining factor. One possible explanation for such promiscuous cleavage sites may be that a certain local secondary structure of the substrate dictates the substrate specificity rather than the primary amino acid sequence. Such structural requirements may explain in part why interaction of polyanionic chondroitin and keratan sulfate chains with non-catalytic ancillary domains of the enzyme influences the activity of ADAMTS-4.

**ADAMTS-4 Forms Found in Cartilage and Biological Functions**—Our study indicates that the substrate specificity of ADAMTS-4 is greatly influenced by its non-catalytic domains. Identification of the 46-kDa ADAMTS-4 as a major form in IL-1α-treated cartilage suggests that the cartilage enzyme expresses strong activity against the Glu295-Ala307 bond as well as the chondroitin sulfate-rich region of aggrecan. In fact, when bovine and porcine cartilage were stimulated by IL-1, GAG is released into the medium along with the ARGSV neoeptope (30, 38). In addition, the 46-kDa form is likely to possess proteolytic activities for other substrates such as fibromodulin and decorin, because both the 53-kDa TS4-2 and the 40-kDa TS4-3 have non-distinguishable activities on these substrates and Cm-Tf. Fibromodulin degradation has been observed in IL-1-treated cartilage, but the enzyme responsible has not been identified (39). The processed form of ADAMTS-4 is clearly a candidate for this activity, and such cleavage of fibromodulin may destabilize collagen fibrils, rendering them more readily susceptible to tissue collagenases. Post-translational processing of full-length ADAMTS-4 is also an important factor in altering localization of the enzyme within cartilage. When the precursor of ADAMTS-4 is synthesized in 293-EBNA cells or chondrosarcoma HTB-94 cells, it is processed intracellularly and secreted as a mature active form of 70 kDa. This full-length form associates with the cell surface and ECM. It is therefore likely that ADAMTS-4 functions pericellularly or near the cells when synthesized and secreted. Recent studies of Pratta et al. (40) also showed that ADAMTS-4 was associated with the surface of chondrocytes and the ECM of cartilage, but the domain responsible for these interactions was not investigated. Our immunolocalization studies with ADAMTS-4 variants showed that the C-terminal Sp domain is responsible for the interaction with the ECM. A number of other ADAMTSs, including ADAMTS-1 (41, 42), ADAMTS-5 (43, 44), and ADAMTS-9 (44) bind to ECM components, but not ADAMTS-13 (45). Kuno and Matsushima (41) reported that the two TS and Sp domains located in the C-terminal end of ADAMTS-1 play a major role in this interaction for mature ADAMTS-1. The loss of the two TS domains also reduces the affinity of ADAMTS-1 to the endothelial cell surface (42). However, when the precursor forms of ADAMTS-1 are secreted from the cell, the prodomain and the catalytic domain are sufficient for binding to the ECM (41). Although full-length ADAMTS-4 binds tightly to the ECM, proteolysis of the C-terminal region of ADAMTS-4 releases the enzyme from the matrix, which then allows it to diffuse into the inter territorial region of the cartilage and degrade aggrecan at the N-terminal interglobular region resulting in the release of larger fragments of aggrecan. IL-1 treatment of cartilage enhanced the production of ADAMTS-4, primarily of the 46-kDa form, which is likely to have strong “classic” aggrecanase activity. These post-translational events must be taken into account when assessing the contribution of ADAMTS-4 during the progression of cartilage degradation in various arthritides.
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Addition and Correction

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Altered proteolytic activities of ADAMTS-4 expressed by C-terminal processing.

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Page 10113, legend to Table I: The last sentence in the legend should read: “One unit of enzyme activity releases 1 μg of GAG/min at 37 °C at pH 7.5.”