TIMP-3 Inhibition of ADAMTS-4 (Aggrecanase-1) Is Modulated by Interactions between Aggrecan and the C-terminal Domain of ADAMTS-4*

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ADAMTS-4 (aggrecanase-1) is a glutamyl endopeptidase capable of generating catabolic fragments of aggrecan analogous to those released from articular cartilage during degenerative joint diseases such as osteoarthritis. Efficient aggrecanase activity requires the presence of sulfated glycosaminoglycans attached to the aggrecan core protein, contributing to the substrate recognition/binding site(s) to ADAMTS-4 activity. In this study, we developed a sensitive fluorescence resonance energy transfer peptide assay with a $K_m$ in the $10 \mu M$ range and utilized this assay to demonstrate that inhibition of full-length ADAMTS-4 by full-length TIMP-3 (a physiological inhibitor of metalloproteinases) is enhanced in the presence of aggrecan. Our data indicate that this interaction is mediated largely through the binding of glycosaminoglycans (specifically chondroitin 6-sulfate) of aggrecan to binding sites in the thrombospondin type 1 motif and spacer domains of ADAMTS-4 to form a complex with an improved binding affinity for TIMP-3 over free ADAMTS-4. The results of this study therefore indicate that the cartilage environment can modulate the function of enzyme-inhibitor systems and could have relevance for therapeutic approaches to aggrecanase modulation.

The disintegrin metalloproteinases with thrombospondin motifs (ADAMTS) are a novel family of extracellular proteases forming an integral part of the extracellular matrix itself. Along with serine proteases, matrix metalloproteinases, bone morphogenetic protein-1/tolloid metalloproteinases, and ADAM (a disintegrin and metalloproteinase) proteins, ADAMTS proteins play a pivotal role in the proteolytic processing and turnover of the component molecules of the extracellular matrix of a broad range of tissues and have potential roles in the turnover of cell-surface proteins. ADAMTS proteins share the characteristic protease, disintegrin-like, and cysteine-rich domains in common with ADAM proteins, but differ in being soluble rather than membrane-bound and by the presence of thrombospondin type 1 (TSP-1) repeats (1, 2). ADAMTS-2, -3, and -14 are potential procollagen N-proteinases, and ADAMTS-13 has been identified as a von Willebrand factor-cleaving protease. ADAMTS-4 is a member of the “angiogenesis/aggre
canase” group of ADAMTS proteases (which also includes ADAMTS-1, -5, -8, -9, and -15) and is unique among the currently known ADAMTS proteases in containing only a single TSP-1-like motif, located between its disintegrin-like and cysteine-rich domains, and lacking any C-terminal TSP-1-like repeats (see Fig. 1). Like the other members of the angiogenesis/aggre
canase group and ADAMTS-9, ADAMTS-4 has been demonstrated to act as an aggrecanase in vitro. In common with other aggrecanases, ADAMTS-4 is able to cleave aggrecan at multiple sites (five in total) (see Fig. 1) (3); however, it is one of only four that cleave aggrecan at the Glu$^{373}$-Ala$^{374}$ “interglobulin domain” cleavage site (the others being ADAMTS-1, -5, and -8, which cleave with varying affinities). Aggrecan is a proteoglycan that is present in articular cartilage and that plays an important role in the ability of cartilage to withstand damage during the mechanical compression of a joint; thus, aggrecanases represent a potential target for the treatment of both osteoarthritis and rheumatoid arthritis (4–6). The efficient cleavage of aggrecan by ADAMTS-4 has been shown to require the presence of both the sulfated glycosaminoglycans (GAGs; chondroitin sulfate and keratan sulfate) attached to the aggrecan core protein and the TSP-1-like motif of ADAMTS-4, which contains a putative GAG-binding site. The treatment of bovine aggrecan to remove keratan sulfate was shown to result in loss of cleavage at the Glu$^{373}$-Ala$^{374}$ cleavage site (7, 8). In contrast, the C-terminal domains of ADAMTS-4 (cysteine-rich/spacer) have been shown to interact directly with the GAG side chains of aggrecan to inhibit cleavage at Glu$^{373}$, Ala$^{374}$, with C-terminal truncation of these domains resulting in markedly increased activity relative to the full-length protein. Indeed, with the spacer domain intact, full-length ADAMTS-4
Aggrecan Regulation of ADAMTS-4/TIMP Interactions

demonstrates only a relatively weak activity at the bond between Glu$^{373}$ and Ala$^{374}$, instead cleaving preferentially at sites in the chondroitin sulfate-rich region (see Fig. 1) (5, 9).

Autocatalytic processing of ADAMTS-4 (68 kDa) has been demonstrated, resulting in the production of two smaller isoforms of 53 and 40 kDa through cleavage within the spacer and cysteine-rich domains, respectively (10). In addition, membrane type 4 matrix metalloproteinase has been shown to remove the spacer domain of ADAMTS-4 (11). It is therefore possible that proteolytic modulation of these interactions may represent a regulatory mechanism in vivo.

Although there is plenty of evidence for the importance of these C-terminal domains in the interactions between ADAMTS-4 and its physiological substrates, no investigation has yet studied their effects on the inhibitory action of the tissue inhibitors of metalloproteinases (TIMP), a series of well-described inhibitors of ADAMTS-4 (12, 13). Of the four currently identified species of TIMP, only TIMP-3 has shown potent inhibition of aggrecanase activity by ADAMTS-4 ($K_i = 7.9$ nM), and TIMP-1 and TIMP-2 are inhibitory at higher concentrations ($K_i \geq 350$ nM) (12). At present, the mechanisms underlying the regulation of TIMP inhibition of ADAMTS-4 in vivo remain undefined. In this study, we propose that TIMP interactions are selectively regulated by electrostatic modification of the C-terminal domains of ADAMTS-4 by aggrecan GAGs, invoking a mechanism independent of the conformational state of the catalytic cleft.

EXPERIMENTAL PROCEDURES

Materials—Unless stated otherwise, all reagents were from Sigma (Poole, UK). Full-length TIMP-1 was produced according to the method described by Murphy et al. (14). Full-length TIMP-3 was purchased from R&D Systems (Abingdon, UK) (15). Both glycosylated and deglycosylated (chondroitinases A, B, and C and keratanases I and II) pig laryngeal aggrecans (domains A1–D1) were kindly provided by Prof. Amanda Fosang (Melbourne University, Melbourne, Australia). Chondroitin 4-sulfate was purchased from Calbiochem (Nottingham, UK), and chondroitin 6-sulfate was from Sigma. Keratan sulfate was purchased from Europa Bioproducts (Ely, UK).

Identification of a Human ADAMTS-4 Substrate from a Phage-displayed Peptide Library and Synthesis of Fluorescent Peptide Substrate—An M13 phage-displayed 10-mer random peptide library (fGWmAb179X10) was constructed by inserting a partially degenerated DNA cassette into Bblsl-digested phage display vector fGW (16). The DNA cassette encoded the monoclonal antibody (mAb) 179 tag (ACLEPYTACD) immediately downstream of the M13 gene III signal sequence and a 10-residue stretch of random amino acids (ACLEPYTACDSAXGGGGS, where X represents any of the 20 common amino acids). Following library phage preparation, a small fraction of transformed Escherichia coli MC1061 cells was plated on LB/tetracycline plates to determine the total number of primary transformants, which was estimated to be $8.5 \times 10^8$. A total of $1 \times 10^{12}$ fGWmAb179X10 phage were digested at $37^\circ$C for 1 h by 10 nM human aggrecanase-1 (ADAMTS-4) in a 250-μl reaction mixture containing 1X aggrecanase-1 buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, and 10 mM CaCl$_2$). The reaction was terminated by the addition of 0.5 mM EDTA to a final concentration of 5 mM. mAb 179 (100 μg) was added to the terminated reaction mixture, followed by the addition of bovine serum albumin to 0.1%. After 30 min of incubation on ice, allowing mAb 179 to capture uncleaved phage, Pansorbin cells (100 μl) were added, and the reaction mixture was rotated at 4°C for 1 h. The mixture was centrifuged for 2 min at 12,000 × g, and the supernatant containing cleaved phage was recovered. The uncleaved phage-mAb complex bound to Pansorbin cells in the pellet was discarded. Phage in the supernatant was transfected into E. coli K91 cells and amplified overnight at 37°C. Phage in a small aliquot of the supernatant was titered on E. coli K91 cells. The above screening was performed for five rounds as described previously except only mAb 179 was used (17). Single phage clones selected by human ADAMTS-4 were picked randomly and grown in 4 ml of LB medium for isolation of DNA for sequencing. A human ADAMTS-4 substrate with a peptide sequence of AELQGRPSIA was identified. This sequence was then used to construct a fluorescence resonance energy transfer (FRET) peptide substrate, carboxyfluorescein (FAM)-ACLEQGRPSIAK-N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), using the FRET pairs fluorescein and tetramethylrhodamine and the protocol described by Pope et al. (18) and Alvarez-Iglesias et al. (19).

Expression and Purification of Recombinant ADAMTS-4—A total of five isoforms of ADAMTS-4 were used in this study (see Fig. 1). The full-length mature form of ADAMTS-4 with a C-terminally His-tagged sequence (TS4-1) was expressed in an Epstein-Barr virus nuclear antigen-expressing human embryonic kidney 293 cell line and purified from conditioned medium by chromatography on a Radial Q column and heparin-Sepharose. Two C-terminal domain deletion enzymes containing both catalytic and disintegrin domains (TS4-4) and the catalytic domain alone (TS4-5) were expressed in Drosophila S2 cells with a C-terminally His-tagged sequence and purified from diaphragm insect cell culture supernatants by nickel-nitri lactric acid (Ni-NTA) chromatography. In addition, a C-terminal domain deletion enzyme containing the catalytic, disintegrin, and thrombospondin domains (TS4-3) with a C-terminal His tag expressed in a baculovirus system and purified from insect cell culture supernatants was purchased from Calbiochem.

Proteolytic Cleavage of ADAMTS-4 to Generate TS4-2* (p53)—The method used to generate TS4-2* (Fig. 1) was based on the method described by Flannery et al. (10) for the proteolytic cleavage of ADAMTS-4. Purified His$_6$-labeled recombinant ADAMTS-4 (68,000 Da) was diluted to 12.5 μg/ml in Tris acetate (pH 7.3), 5 mM CaCl$_2$, and 100 mM NaCl and incubated at 37°C for 64 h. Reaction products were purified through multiple Ni-NTA spin columns (Qiagen Inc., Crawley, UK) to remove uncleaved protein. Proteolytically cleaved ADAMTS-4 was detected by SDS-PAGE and Western immunoblotting with mAb 16297 (Abcam, Cambridge, UK). A single C-terminally truncated isoform of ADAMTS-4 (TS4-2*), approximating in size that described for ADAMTS-4 (p53) by Flannery et al. (10) and representing a close equivalent to the TS4-2 construct described by Kashiwagi et al. (9), was confirmed (Fig. 2A). Matrix-assisted laser desorption ionization (MALDI) mass
Aggrecan Regulation of ADAMTS-4/TIMP Interactions

A

FIGURE 1. Domain structures of ADAMTS-4 and aggrecan. A, the domain structures of ADAMTS-4 truncates tested in this study. TS4-1 represents the full-length mature form ADAMTS-4. The asterisk indicates the cleavage site for TS4-2\* proposed by Flannery et al. (10). B, aggrecanase cleavage sites in human aggrecan. HA, hyaluronic acid; L, link protein; IGD, interglobular domain; KS, keratan sulfate-enriched region; CS1 and CS2, chondroitin sulfate-enriched regions 1 and 2; G1, G2, and G3, globular domains 1–3.

B

FIGURE 2. Production of the ADAMTS-4 isoform TS4-2\* from the full-length enzyme. A, SDS-PAGE/Western blot analysis. Purified ADAMTS-4 was adjusted to a concentration of 12.5 μg/ml (lane 1, upper band) and incubated at 37 °C for 64 h (lane 2). This sample also contained a co-purified N-terminally truncated isoform of ADAMTS-4 lacking a functioning catalytic domain (identity confirmed by peptide mass fingerprinting using MALDI mass spectroscopy) (lane 1, lower band). Reaction products were purified through sequential Ni-NTA spin columns to produce the purified TS4-2\* isoform (lane 2). 8, amino acid sequence of the C-terminal cysteine-rich and spacer domains of ADAMTS-4 showing (i) the most C-terminal amino acid residue confirmed in TS4-2\* by MALDI mass spectroscopy sequence assignment (arrow) and (ii) the autocatalytic cleavage site described by Flannery et al. (10) (arrowhead).

eq real-time increases in fluorescence intensity, at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Bio-Tek Instruments FL600 microplate spectrophotometer.

Inhibitor samples were diluted in 50 mM Tris-HCl (pH 7.5) and 12.5% glycerol and dispensed into Nunc black polystyrene 384-well microtiter plates (VWR International, Leicestershire, UK) in a volume of 10 μl. ADAMTS-4 and FAM-AELQGRPIISAK-TAMRA peptide were diluted 50 mM BisTris propane (pH 8.0), 150 mM NaCl, 10 mM CaCl2, and 0.01% Triton X-100, and 20 μl was added to the inhibitor plate such that their final assay concentrations were 200 μM and 0.5 μM, respectively. Peptide hydrolysis was measured for 4 h at 20 °C.

Apparent Kᵢ values were determined using the Robosage Version 7.31 add-in (GlaxoSmithKline, Research Triangle Park, NC) for Excel (Microsoft Corp., Redmond, WA) and the four-parameter logistic equation y = (A – D)/(1 + (x/C⁻¹)) + D, where x is the concentration of the test sample, y is the percent inhibition, A is the minimum response, D is the maximum response, C is the apparent Kᵢ (Kᵢₐₚₙₜ or IC₅₀) for the curve, and B is the Hill slope. Mean values for Kᵢₐₚₙₜ were determined from a fit of the mean percentage inhibition values, at each concentration of inhibitor, to the four-parameter logistic equation. The S.E. of the curve fit (S.E. of the estimate) was determined from the average scatter of the points from the fitted curve using the Robosage Version 7.31 curve-fitting tool.

Kinetic analyses were performed with GraFit Version 5.0 software (Erithacus Software Ltd., Staines, UK). The initial velocities (V) of substrate hydrolysis were determined at various substrate concentrations ([S]; 0–25 μM) and used to estimate the Michaelis (Kₘ) and catalytic (kₐₜ) constants by applying the equations V = Vₕₐₙₓ[S]/([S] + Kₘ) and kₐₜ = Vₕₐₙₓ/E, where Vₕₐₙₓ describes the estimated maximum velocity and [E]
describes the enzyme concentration. In determining \( K_m \) values, the effects of internal quench due to inner filter from the FRET peptide substrate on the kinetic rates determined were corrected for by calibration against a fluorescein-labeled peptide control (20).

Association and dissociation rate constants were determined by continuous monitoring of peptide cleavage by 400 pM ADAMTS-4 using GraFit Version 5.0 software. Initial velocities \( (V_o) \) and steady-state velocities \( (V) \) of substrate hydrolysis were determined at various concentrations of TIMP-3 \((0 - 40 \text{ nM})\) and used to estimate the association \( (k_{on}) \) and dissociation \( (k_{off}) \) rate constants by applying the equations \( F = \left( V(t) + (V_o - V(t))/(1 - e^{(-k_{off}/k_{on})}) \right) + F_0 \) and \( k_{obs} = k_{on}[I] + k_{off} \) where \( F \) is the observed fluorescence, \( F_0 \) is the initial fluorescence, \( k_{obs} \) is the observed rate constant, \( t \) is time, and \[ I \] is the inhibitor concentration.

**RESULTS**

Cleavage of the FAM-TAMRA Peptide by Recombinant ADAMTS-4 and Truncated Isoforms—The quenched fluorescent peptide FAM-AELQGRPISIAK-TAMRA was cleaved by recombinant ADAMTS-4 (TS4-1) at a single site to produce the fragments FAM-AE and LQGRPISIAK-TAMRA, confirming the glutamyl endopeptidase nature of this enzyme. In the presence of recombinant ADAMTS-4 (TS4-1), a 3-fold increase above the background fluorescent signal was observed over 240 min. The reaction curve was linear over time with the rate of peptide cleavage effectively constant. No peptide cleavage was observed in the absence of recombinant ADAMTS-4, and complete inhibition of the cleavage was achieved by the addition of the hydroxamate derivative matrix metalloproteinase inhibitor CGS27023 (100 M; GlaxoSmithKline, Stevenage, UK) (12). A pH optimum of 8.0 was determined for ADAMTS-4 in this assay (data not shown). Under our optimized conditions, \( k_{cat}/K_m \) values averaging \( 1 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) were measured (Table 1). The assay was highly sensitive, with peptide hydrolysis by as little as 50 pM ADAMTS-4 producing excellent \( z' \) values (averaging 0.85), confirming that the assay was statistically highly reproducible (21). TS4-5 was catalytically inactive, in agreement with previously published findings (9). However, the isoforms TS4-2*, -3, and -4 all demonstrated measurable rates of peptide hydrolysis at subnanomolar concentrations, and \( K_m \) and \( k_{cat}/K_m \) values similar to those observed using the full-length ADAMTS-4 construct were observed (Table 1). In each case, complete inhibition of the cleavage was achieved by the addition of 100 M CGS27023 (22).

TIMP Inhibition of ADAMTS-4 Using the FAM-TAMRA Peptide Assay—Using the conditions described above, we investigated the effects of the addition of TIMP-3, TIMP-1, and the broad-spectrum metalloproteinase inhibitor CGS27023 to TS4-1 in the FAM-TAMRA peptide assay. A \( K_i \) of 6.38 \pm 1.51 nM was determined for TIMP-3. This compares with a published value of 7.9 nM using aggrecan as the substrate (12). Little or no inhibition of TS4-1 was observed with TIMP-1 \((K_i > 350 \text{ nM})\), and a \( K_i \) of 0.87 \pm 0.16 M was determined for CGS27023.

Aggrecan Enhances TIMP-3 Potency—We investigated the effect of the potential physiological substrate aggrecan on the interaction between ADAMTS-4 and TIMP-3 using the FAM-TAMRA peptide assay. The addition of 10 M aggrecan to the assay in the absence of TIMP-3 resulted in an ~50% inhibition of peptide hydrolysis due to competitive inhibition by the presence of an alternative substrate at a concentration close to its Michaelis constant (23). The \( K_m \) and \( k_{cat} \) values for ADAMTS-4 determined in the presence of 10 M aggrecan were also consistent with expectations for a purely competitive inhibition of the alternative substrate aggrecan with respect to peptide cleavage (Table 2). Interestingly, the presence of aggrecan resulted in an increase in the potency of TIMP-3, with an order of magnitude increase in potency observed at aggrecan concentrations > 1 M (Fig. 3).

In contrast, CGS27023 inhibition was unaffected by the presence of 10 M aggrecan, with a \( K_{i(appo)} \) of 1.23 \pm 0.24 M being measured. As before, no activity was measured for TIMP-1 under these assay conditions \((K_i) > 350 \text{ nM})\). Association and disso-

### TABLE 1

| ADAMTS-4 truncate | \( K_m \) \( \mu M \) | \( k_{cat}/K_m \) \( M^{-1} \text{s}^{-1} \) |
|-------------------|----------------|------------------|
| TS4-1             | 10.7           | 8.02E+04         |
| TS4-2*            | 16.2           | 1.72E+05         |
| TS4-3             | 15.4           | 4.57E+04         |
| TS4-4             | 13.5           | 1.41E+05         |

| ADAMTS-4 inhibition | \( I_a \) |
|---------------------|----------|
| Control              | 1.07     |
| Aggrecan             | 1.16     |
| Chondroitin sulfate  | 1.49     |

![FIGURE 3. Effects of aggrecan on TIMP-3 inhibition of ADAMTS-4.](image)

**TABLE 2**

Effects of aggrecan and chondroitin sulfate on ADAMTS-4 \( k_{cat} \) and \( K_m \)

FRET peptide hydrolysis by 1 nM ADAMTS-4 (TS4-1) measured over a 20-min (linear) period was used in the determination of \( k_{cat} \) and \( K_m \) values under normal assay conditions, as well as in the presence of 10 \mu g/ml aggrecan and 100 \mu g/ml chondroitin 4-sulfate. 100 mM NaCl was used in the assay. \( I_a \) inhibition in the absence of substrate determined by linear fit. Data are the mean data \( (n = 3) \).
ciation constants for TIMP-3 inhibition of ADAMTS-4 were also determined in the presence and absence of aggrecan and demonstrated that the addition of aggrecan resulted in an increase in the observed $k_{\text{on}}$, but had little effect on $k_{\text{off}}$ (Table 3).

**Enhancement of TIMP-3 Potency by Aggrecan Is Mediated through GAGs**—Having ascertained that chondroitin sulfate did not significantly modify the $K_m$ and $k_{\text{cat}}$ values for ADAMTS-4 turnover of the FRET peptide (Table 2), we monitored its effect on TIMP-3 interactions with ADAMTS-4 using this assay. Whereas chondroitin 4-sulfate had little effect on $K_i$ values, chondroitin 6-sulfate enhanced TIMP-3 binding to ADAMTS-4 at low doses (Fig. 4). Chondroitin 4-sulfate also had the effect of interfering significantly with the aggrecan potentiation of TIMP-3 inhibition, whereas chondroitin 6-sulfate had less effect. Keratan sulfate demonstrated no determinable effect on TIMP-3 inhibition of ADAMTS-4 in either the presence or absence of aggrecan (Fig. 5). The effects of deglycosylating aggrecan on its enhancement of TIMP-3 potency were also studied, and in line with the results obtained in studies of chondroitin sulfates, deglycosylated aggrecan was markedly less efficient in the modulation of TIMP-3 potency (Fig. 6).

**Aggrecan Modulation of TIMP-3 Potency Is Dependent on the ADAMTS-4 C-terminal Domain**—TIMP-3 inhibition of the four C-terminally truncated isoforms of ADAMTS-4 was studied in the absence and presence of aggrecan (Fig. 1). Under normal assay conditions, all the forms gave similar binding constants with TIMP-3, although the TS4-4 construct containing the catalytic and disintegrin-like domains showed a small enhancement of affinity for TIMP-3 (Table 4). In the presence of aggrecan, only TS4-1 showed a substantial (20-fold) increase in TIMP-3 affinity. The data indicate that the His tag present in all the TS4 forms except TS4-2* had no effect on TIMP-3 or aggrecan interactions.

**The Ionic Environment Modulates TIMP-3 Potency**—To investigate whether the interactions observed between ADAMTS-4, aggrecan, chondroitin sulfate, and TIMP-3 could be electrostatic in nature, the experiments described above were repeated using assay buffer lacking NaCl. At low ionic strength, neither hydrolysis of the FAM-TAMRA peptide substrate nor the potency of CGS27023 inhibition was significantly

### TABLE 3

|          | No aggrecan | 10 μg/ml aggrecan |
|----------|-------------|-------------------|
| $k_{\text{on}}$ (s$^{-1}$) | 9217 ± 2216 | 462,857 ± 89,076 |
| $k_{\text{off}}$ (s$^{-1}$) | 0.0006 ± 0.00005 | 0.0004 ± 0.0001 |

**FIGURE 4.** Effects of chondroitin 4-sulfate and chondroitin 6-sulfate on TIMP-3 inhibition of ADAMTS-4. A, effects on TIMP-3 potency of chondroitin 6-sulfate (shaded bars) and chondroitin 4-sulfate (black bars) ($n = 10$). B, interactions between chondroitin 4-sulfate (C-4-S), chondroitin 6-sulfate (C-6-S) and aggrecan. Aggrecan (10 μg/ml) or chondroitin sulfate (100 μg/ml) was used in the determination of $K_{\text{app}}$ values ($n = 3$). Significance testing of paired $p_{K_{\text{app}}}$ data demonstrated statistically significant enhancement of TIMP-3 potency by aggrecan ($p = 2.99 × 10^{-7}; n = 10$) and chondroitin 6-sulfate ($p = 3.16 × 10^{-5}; n = 5$) as well as interference of aggrecan-enhanced TIMP-3 potency by chondroitin 4-sulfate ($p = 7.88 × 10^{-6}; n = 5$). Data are the $K_{\text{app}}$ values of the mean data ± S.E. of the curve fit.

**FIGURE 5.** Effects of aggrecan and keratan sulfate on TIMP-3 inhibition of ADAMTS-4. Hydrolysis of the FRET peptide in assay buffers containing aggrecan (10 μg/ml) and/or keratan sulfate (KS) was used in the titration of TIMP-3. Data are the $K_{\text{app}}$ values of the mean data ± S.E. of the curve fit ($n = 2$).
Effects of ADAMTS-4 C-terminal domains on the interaction between ADAMTS-4, TIMP-3, and aggrecan

The mean rates of hydrolysis over 4 h were used in the determination of $K_{\text{app}}$ values. Data are the $K_{\text{app}}$ values of the means ± S.E. of the curve fit ($n \geq 3$).

| [Aggrecan] | [NaCl] | $K_{\text{app}}$ | S.E. | $K_{\text{app}}$ | S.E. | $K_{\text{app}}$ | S.E. | $K_{\text{app}}$ | S.E. |
|-----------|--------|----------------|-----|----------------|-----|----------------|-----|----------------|-----|
| 0 µg/ml   | 0      | 6.38           | 1.51| 5.72           | 3.75| 6.17           | 1.44| 3.63           | 0.25|
| 10 µg/ml  | 100    | 0.34           | 0.05| 2.64           | 0.55| 3.3            | 0.39| 3.13           | 0.20|
| 0 µg/ml   | 0      | 0.80           | 0.11| 0.68           | 0.15| 13.09          | 4.29| 5.07           | 0.47|
| 10 µg/ml  | 0      | 0.21           | 0.04| 0.07           | 0.01| 0.36           | 0.04| 2.54           | 0.14|
chondroitin 6-sulfate side chains of aggrecan, but may also involve a contribution from the aggrecan core protein.

The differences between the effects of chondroitin 6-sulfate and keratan sulfate may also suggest a possible partial explanation as to why the interglobular domain is so susceptible to proteolytic cleavage by aggrecanases. However, it should also be noted that, in this study, we investigated only a single form of keratan sulfate, which has been reported previously to undergo both age-related changes in chain length, sulfation, fucosylation, and sialic acid capping (26) and to vary in structure between the keratan sulfaterich region and interglobular domain of aggrecan (25). Therefore, it remains a possibility that alternative forms of keratan sulfate may demonstrate properties different from those observed in this study.

Investigation of the mechanisms underlying the interactions observed demonstrated that aggrecan behaved as a purely competitive inhibitor of ADAMTS-4, whereas chondroitin sulfate had no effect. Little or no effect on the rate of dissociation of TIMP-3 from ADAMTS-4 was seen; however, a large increase in the TIMP-3 association rate constant was observed in the presence of aggrecan. The lack of change in apparent affinity of the low molecular mass inhibitor CGS27023 in the presence of aggrecan suggests that this effect is not mediated through a conformational change in the catalytic cleft, but results in enhanced TIMP-3 potency possibly by facilitating its binding to ADAMTS-4. Furthermore, the resulting enzyme-inhibitor complex is not affected by aggrecan because the first-order dissociation constant remains unchanged, thus ruling out any significant mechanism preventing the release of TIMP-3 from ADAMTS-4, once bound.

GAG/protein-binding interactions are electrostatic in nature (27), and we were able to demonstrate that the potency of TIMP-3 was enhanced by reducing the ionic strength of our assay buffer. GAG-binding sites on TIMP-3 have been postulated previously since it was shown to bind heparan sulfate and chondroitin sulfate (28). Similarly, the cysteine-rich, TSP-1, and spacer domains of ADAMTS-4 also contain GAG-binding motifs, which have been shown to interact with the GAG chains of aggrecan (5, 10). In contrast to full-length ADAMTS-4 (TS4-1), studies of a selection of truncated forms of ADAMTS-4 (TS4-2*, -3, and -4) demonstrated that, at physiological ionic strength, TIMP-3 inhibition of ADAMTS-4 constructs lacking the C-terminal spacer domain was largely unaffected by the presence of aggrecan. The aggrecan-associated enhancement of TIMP-3 potency was restored in constructs containing an intact TSP-1 domain (TS4-2* and TS4-3) when NaCl was omitted from assay buffers; however, TIMP-3 inhibition of TS4-4 remained constant irrespective of NaCl or aggrecan concentrations. Significantly, the equivalent responses of the His-tagged TS4-3 and tag-free TS4-2* truncates to the addition of aggrecan under these conditions serve to rule out the possibility that the presence of a His tag is responsible for these responses. Taken together, these results suggest that the observed aggrecan-dependent enhancement of TIMP-3 potency is mediated through interactions at the TSP-1-like motif of ADAMTS-4, but that, under normal physiological conditions, additional binding interactions between the GAG side chains of aggrecan and the spacer domain of ADAMTS-4 are also necessary to facilitate binding at the TSP-1-like motif.

From the results of this study, we conclude that TIMP-3 interaction with ADAMTS-4 is mediated through the binding of GAGs (specifically chondroitin 6-sulfate) of aggrecan to the TSP-1 motif and spacer domains of ADAMTS-4 to form a complex with an improved binding affinity for TIMP-3 over free ADAMTS-4 either through the allosteric modulation of a TIMP-binding exosite of ADAMTS-4 or through the formation of a TIMP-enzyme-chondroitin 6-sulfate complex with chondroitin 6-sulfate serving as a scaffold to facilitate TIMP-3 presentation at the catalytic cleft. It has been shown that the accelerating effect of heparin on the binding of antithrombin to factor Xa is at least partly due to heparin promoting the ordered assembly of antithrombin and factor Xa in an intermediate ternary complex (29). Interestingly, although a previous study has described potential GAG-binding interactions between TIMP-3 and GAGs within the extracellular matrix (28), our own study investigating TIMP-3-binding interactions with aggrecan found no evidence for direct binding interactions, suggesting that allosteric modulation may present a more probable model.

Within physiological contexts, the unique environment created by the concentration of sulfated sugars associated with different proteoglycans such as aggrecan could act as a significant regulator of both enzyme and inhibitor activity. The efficacy of TIMP-3 interaction with ADAMTS-4 in cartilage could be modulated by changes in the ratio of chondroitin 4-sulfate to chondroitin 6-sulfate (24). In other tissues with less GAG content, TIMP-3 would be a considerably less efficient inhibitor of ADAMTS-4. Knowledge of the precise binding sites of both chondroitin 6-sulfate and TIMP-3 on the exodomains of ADAMTS-4 will provide useful information for the design of highly specific inhibitors for this enzyme.

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Aggrecan Regulation of ADAMTS-4/TIMP Interactions

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