**α₂-Macroglobulin Is a Novel Substrate for ADAMTS-4 and ADAMTS-5 and Represents an Endogenous Inhibitor of These Enzymes**

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Osteoarthritis is characterized by the loss of aggregan and collagen from the cartilage extracellular matrix. The proteinases responsible for the breakdown of cartilage aggrecan include ADAMTS-4 (aggrecanase 1) and ADAMTS-5 (aggrecanase 2). Post-translational inhibition of ADAMTS-4/-5 activity may be important for maintaining normal homeostasis of aggrecan metabolism, and thus, any disruption to this inhibition could lead to accelerated aggrecan breakdown. To date TIMP-3 (tissue inhibitor of matrix metalloproteinases-3) is the only endogenous inhibitor of ADAMTS-4/-5 that has been identified. In the present studies we identify α₂-macroglobulin (α₂M) as an additional endogenous inhibitor of ADAMTS-4 and ADAMTS-5. α₂M inhibited the activity of both ADAMTS-4 and ADAMTS-5 in a concentration-dependent manner, demonstrating 1:1 stoichiometry with second-order rate constants on the order of 10³ and 10⁵ M⁻¹ s⁻¹, respectively. Inhibition of the aggrecanases was mediated by proteolysis of the bait region within α₂M, resulting in physical entrapment of these proteinases. Both ADAMTS-4 and ADAMTS-5 cleaved α₂M at Met⁴⁰⁰⁹/Gly⁴⁰¹, representing a novel proteinase cleavage site within α₂M and a novel site of cleavage for ADAMTS-4 and ADAMTS-5. Finally, the use of the anti-neoepitope antibodies to detect aggrecanase-generated α₂M-fragments in synovial fluid was investigated and found to be uninformative.

Loss of aggrecan from the cartilage extracellular matrix is an early and sustained feature of osteoarthritis (OA). Aggrecan, the major proteoglycan in cartilage, consists of a protein backbone of 210–250 kDa containing 3 globular domains referred to as G1 (located at the N terminus), followed by G2 and G3 (located at the C terminus) (1). Attached to the core protein between G2 and G3 are the glycosaminoglycans, chondroitin sulfate, and keratan sulfate. The chondroitin sulfate chains (100–125 per monomer) are located in the C-terminal portion of the core protein, whereas the keratan sulfate chains (25–50 per monomer) are preferentially located toward the N terminus (2). Multiple aggrecan monomers interact with hyaluronic acid via their G1 domain to form aggregates of very high molecular weight. The negatively charged glycosaminoglycan chains are responsible for the extremely high osmotic swelling pressure of cartilage, which is counteracted by the resistance of type II collagen fibers (the other major macromolecule of cartilage). Thus, aggrecan provides cartilage with the ability to resist compressive forces, and its loss will have a severe effect upon the functionality of the cartilage.

The cleavage of cartilage aggrecan in OA has been attributed primarily to aggrecanase 1 and 2 (3–9). These enzymes are metalloproteinases that belong to the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family and have been designated ADAMTS-4 and ADAMTS-5, respectively. Both enzymes cleave the core protein of aggrecan after the amino acids Glu³⁹³ and Glu⁴⁴¹, respectively (10, 11). Because of their preference for cleaving at the C terminus of glutamic acid, these enzymes have been referred to as glutamyl endopeptidases (12). In fact, it has been shown that ADAMTS-4 can cleave other chondroitin sulfate proteoglycans, including brevican and versican after Glu³⁹³ and Glu⁴⁴¹, respectively (13–15).

Tight regulation of aggrecanase activity is critical for maintaining a fine balance between aggrecan anabolism and catabolism. In diseases such as OA the balance is disturbed in favor of catabolism, and this could be attributed to de novo synthesis of ADAMTS-4 (9, 16) and/or post-translational activation of ADAMTS-4/-5 (17, 18). Another control mechanism for aggrecan catabolism may involve endogenous inhibitors of the aggrecanases. Recently it has been shown that tissue inhibitor of matrix metalloproteinases-3 (TIMP-3) is a potent inhibitor with Kᵢ(app) values of 3.30 nm for ADAMTS-4 and 0.66 nm for ADAMTS-5 (19, 20). TIMP-3 was found to have a greater affinity for ADAMTS-4 and ADAMTS-5 than for MMP-1, MMP-2, and MMP-3, suggesting that a primary physiological function for TIMP-3 is inhibition of the aggrecanases (19). Moreover, TIMP-3 has been detected in cartilage (21) and shown to have a high binding affinity for chondroitin sulfate polysaccharides, a binding affinity not found in the other 3 members of the family (TIMP-1, -2, -4) (22, 23). Whether TIMP-3 is a physiologically relevant inhibitor of aggrecanase activity in cartilage has yet to be established. Recently, Mort et al. (24) show that aggrecan turnover involving MMP and aggrecanase activity are not under effective TIMP-3 control in an inflammatory arthritis model induced in TIMP-3 null mice, as both wild type and TIMP-3 −/− mice showed similar long term cartilage destruction (24). These data suggest that other inhibitors may be important in the regulation of aggrecanase activity. To date no

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The abbreviations used are: OA, osteoarthritis; MMP, matrix metalloproteinases; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; α₂M, α₂-macroglobulin; TIMP-3, tissue inhibitor of matrix metalloproteinases-3; SF, synovial fluid.
other protein inhibitors of ADAMTS-4 and ADAMTS-5 have been identified. α-Macroglobulin (αM), a general endoproteinase inhibitor, is a noncovalent tetramer of two 370-kDa disulfide-linked homodimers that circulate in blood at concentrations of 2–4 mg/ml and is also found in the joint fluid at similar concentrations (25, 26). αM is active against most endoproteinases. Each subunit of the αM molecule contains a region referred to as the "bait region," a short stretch of amino acids (beginning at Pro667 and ending at Thr705) that is very susceptible to proteolytic cleavage. When cleaved by an enzyme the macroglobulin changes shape in such a way as to trap the proteinase inside, resulting in inhibition of proteolytic activity by preventing substrate access to the active site of the proteinases through steric hindrance (27–29). The conformational changes initiated by bait region cleavage also cause activation of internal thiol esters formed from Cys949 and Glx952 in each subunit of αM (30). The activated thiol esters provide αM with a potential for covalent cross-linking of the activating proteinase through ε-lysyl (proteinase)-γ glutamyl-α(M) bonds and also for binding of other nucleophiles present at activation (31, 32). αM does not inhibit endoproteinases that are highly specific for one or a limited number of sequences, such as tissue kallikrein, urokinase, coagulation factor XIIa, and endoproteinase Lys-C (27). Whether αM can inhibit ADAMTS-4 and ADAMTS-5 activity by proteolysis of the bait region has not been investigated, although it has been shown that a related enzyme, ADAMTS-1, which can cleave purified aggrecan in vitro in the IGD at Glu773, forms an SDS-stable complex with αM (14, 33). Analysis of the 39 amino acids representing the bait region reveals 5 glutamic acids, Glu673, Glu678, Glu686, Glu701, and Glu702, which can cleave purified aggrecan. The time course of inactivation at each concentration of 2M was fit to a single exponential to yield observed inactivation rates of 0.109 and 0.330 min−1 for 0.5 nM ADAMTS-4 in the presence of 25 and 75 nM 2M, respectively. The second-order rate constants for ADAMTS-4 and -5 cleavage of aggrecan at Glu773 by Western blot analysis using the 1772AGEG neoepitope antibody. The densitometric response was found to be linear over the density ranges required for the blots as assessed by loading varying amounts of AGE product and quantifying the immunoreactive bands by scanning densitometry using the software OneDscan. Actice site concentrations were determined by fitting the simple second-order model using the numerical integration software package, DYNAFIT (38) using the nonlinear curve-fitting program "the nonlinear regression software package GraFit" (Erithacus Software Ltd., Staines, UK) was used, and the second-order rate constants were fixed at 105 and 106 M−1 s−1 for ADAMTS-4 and -5, respectively.

**Time-dependent inhibition of ADAMTS-4/-5 by αM**—45 μl of 2 × buffer (100 mM Tris-HCl, 20 mM CaCl2, and 200 mM NaCl, pH 7.5) containing ADAMTS-4 or -5 followed by 25 μl of buffer containing 5 μM Tris, 10 mM NaCl, pH 7.5) containing αM at a range of concentrations (concentrations based on the M, of the two enzyme samples for 2 h at 37 °C. The concentration of aggrecan was determined in the dimethylthelylene blue assay as previously described (36). Final concentrations in the reaction mixture were 25 nM ADAMTS-4 or ADAMTS-5, 500 nM aggrecan, and αM at 0, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, and 150 nM. Reactions were quenched by adding EDTA to a final concentration of 50 mM. The products were analyzed for cleavage of aggrecan at Glu773 by Western blot analysis using the 1772AGEG neoepitope antibody. The densitometric response was found to be linear over the density ranges required for the blots as assessed by loading varying amounts of AGE product and quantifying the immunoreactive bands by scanning densitometry using the software OneDscan. Actice site concentrations were determined by fitting the simple second-order model using the numerical integration software package, DYNAFIT (38) using the nonlinear curve-fitting program "the nonlinear regression software package GraFit" (Erithacus Software Ltd., Staines, UK) was used, and the second-order rate constants were fixed at 105 and 106 M−1 s−1 for ADAMTS-4 and -5, respectively.

**Calculation of Second Order Rate Constants**—Time-dependent inhibition data was analyzed by several different methods to estimate second-order rate constants for the reaction between αM and ADAMTS-4 and -5. The time course of inactivation at each concentration of enzyme and αM was fit to a simple exponential to yield observed inactivation rates of 0.109 and 0.330 min−1 for 0.5 nM ADAMTS-4 in the presence of 25 and 7.5 nM αM and 0.115 and 0.328 min−1 for 5 nM ADAMTS-5 in the presence of 25 and 75 nM αM, respectively. The linear dependence of inactivation rate on αM concentration suggested that at these concentrations it was proper to analyze this reaction as a simple first-order process. Dividing these observed rates by the αM concentration gave estimates for the second-order rate constants for ADAMTS-4 and -5, respectively. Inactivation data was also fit globally using the numerical integration software package, DYNAN2T (38) using the simple second-order model. Calculation of second-order rate constants for ADAMTS-4 and -5, respectively. Finally, because of the scarcity of data points during the 0–10 min time range when most of the inactivation took place, the second-order rate constant was calculated directly (i.e. without curve fitting) from the extent of inactivation observed at 5 and/or 10 min using the equation $k = \ln([E]/[EI])/([E]/[EI])E = ([E]_0 - [E]/[EI])$. In the second-order reaction, [E] is the total enzyme concentration, [I] is the total αM concentration, and [EI] is the concentration of enzyme-αM complex at time t (37).

**Inactivation of αM by Methyleneamine**—10 mg/ml αM was incubated with 100 mM sodium borate, pH 8.6, in the presence or absence of 400 mM methyleneamine for 2 h at 37 °C (39). Subsequently, the untreated and
methylamine-treated α-M were tested for their ability to inhibit ADAMTS-4 and ADAMTS-5.

**Cleavage of α-M by ADAMTS-4—ADAMTS-4 and ADAMTS-5 at a concentration of 200 or 112 nM, respectively, were incubated with 250 nM α-M in buffer containing 50 mM Tris-HCl, 100 mM NaCl, and 10 mM CaCl₂, pH 7.5, for 4 h at 37 °C. Subsequently, the products were analyzed for total protein by SDS-PAGE on 8% gels followed by staining with Coomassie Brilliant Blue R-250.**

**Cleavage of α-M by Truncated ADAMTS-4—250 nM α-M was incubated with 200 nM mature truncated ADAMTS-4 (Phe₁₋⁻Phe₁⁻) in buffer containing 50 mM Tris, 100 mM NaCl, 10 mM CaCl₂, pH 7.5, for 2 h at 37 °C. After the digests cleaved α-M was detected by SDS-PAGE using an 8% gel followed by Coomassie staining. Truncated ADAMTS-4 cleaves the C-terminal heparin sulfate-proteoglycan-like domain and the E691 domain of α-M.**

**N-terminal Sequencing—** α-M or aggrecan product for each sample was loaded on a 12.5% SDS-PAGE gel, separated by SDS-PAGE under reducing conditions. The separated proteins were transferred to polyvinylidene difluoride membranes and immunolabeled with a 1:1000 dilution of YESDVM₁₀⁰⁻GRGHAR (for detecting α-M) or 1772AGEG for detecting aggrecan) neoepitope antibody. Subsequently, the membranes were incubated with a 1:5000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate as the secondary antibody. Products were visualized by developing the blots in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color developing reagent. Overnight transfer resulted in complete transfer of both low and high molecular fragments, and the densitometric response was found to be linear over the density ranges required for the blots.

**Detection of the GRGH Neoepitope in Osteoarthritic Synovial Fluid**—Synovial fluids were collected from patients with radiographic and symptomatic OA. Synovial fluids were diluted 100-fold with buffer containing 50 mM Tris, 100 mM NaCl, 10 mM CaCl₂, pH 7.5, and either analyzed for the presence of the GRGH neoepitope by Western blot analysis or incubated with ADAMTS-4 at a concentration of 50 nM for 1 h at 37 °C. After the reactions cleavage of endogenous α-M at Met⁶⁹⁵ was monitored by Western blot analysis using the polyclonal antibody against α-M or the 1772AGEG neoepitope antibody.

### RESULTS

**Inhibition of ADAMTS-4 and ADAMTS-5 by α-M**—We first examined the ability of α-M to block ADAMTS-4 and ADAMTS-5 cleavage of aggrecan. ADAMTS-4 or ADAMTS-5 at a concentration of 25 nM (determined by active site titration with TIMP-3) were preincubated with α-M at varying concentrations for 2 h at 37 °C. After the preincubation aggrecan was added to a final concentration of 500 nM, and the reactions were carried out for 30 min at 37 °C. The samples were subsequently analyzed for aggrecan products by cleavage at the Glu¹⁷⁷¹/Ala¹⁷⁷² bond by Western blot analysis using the 1772AGEG neoepitope antibody. α-M effectively inhibited both ADAMTS-4 and ADAMTS-5 cleavage of aggrecan in a concentration-dependent manner (Fig. 1, A and B), showing almost complete inhibition at 100 and 150 nM, respectively. The concentration of ADAMTS-4 and ADAMTS-5 determined by active site titration with α-M (14 and 57 nM, respectively) are in alignment with the concentrations determined by TIMP-3 titration (25 nM).

**Time-dependent Inhibition of ADAMTS-4 and ADAMTS-5 by α-M**—ADAMTS-4 at a concentration of 0.5 nM was preincubated with α-M at a concentration of 2.5 and 7.5 nM for varying periods of time at 37 °C. After the preincubation aggrecan was added to a final concentration of 500 nM, and the reactions were incubated for 30 min at 37 °C. The products were analyzed by Western blot analysis using the 1772AGEG neoepitope antibody. Inhibition of ADAMTS-4 (Fig. 2) was concentration- and time-dependent. No inhibition of aggrecan cleavage at the Glu¹⁷⁷¹/Ala¹⁷⁷² bond was observed when α-M was not preincubated with ADAMTS-4, but after a 10-min preincubation ~100 and ~68% inhibition was achieved with α-M at concentrations of 2.5 and 7.5 nM, respectively. Similar results were obtained when the inhibition of 5 nM ADAMTS-5 by 25 and 75 nM α-M was examined (data not shown). A variety of approaches yielded estimates for the second-order rate constants for the reaction of α-M with ADAMTS-4 and ADAMTS-5 to be on the order of 10⁵ and 10⁶ M⁻¹ s⁻¹, respectively (see “Experimental Procedures”).

**ADAMTS-4 and ADAMTS-5 Cleave α-M—** α-M inhibits most proteases by physical entrapment upon cleavage within the bait region by the enzyme. This process is disturbed by the treatment of α-M with methylamine, which breaks several key thioester bonds important to the structural integrity of the macroglobulin molecule. After breakage of these bonds the protein undergoes conformational changes, resulting in a less exposed bait region, thus inactivating the “trapping” mechanism (40). Methylamine-treated α-M failed to inhibit ADAMTS-4 and ADAMTS-5 (data not shown), suggesting that the inhibition of these two proteases by α-M is triggered by proteolysis of the bait region and not through allosteric binding and/or steric hindrance.

To confirm that α-M is cleaved by ADAMTS-4 and ADAMTS-5, 250 nM α-M was incubated with 200 nM ADAMTS-4 or 112 nM ADAMTS-5 for 2 h at 37 °C, and the products were separated by SDS-PAGE on an 8% gel and analyzed for total protein by Coomassie staining. Intact α-M in its monomeric form was detected at a molecular mass of ~180 kDa. In the presence of ADAMTS-4 most of the intact α-M was converted to a lower molecular mass species of ~98 kDa (Fig. 3A). In the presence of ADAMTS-5 about 50% of intact α-M was converted to the 98-kDa species (Fig. 3A). These levels of conversion are consistent with 1:1 stoichiometry between inhibitor and enzyme. To confirm that cleavage of α-M by ADAMTS-4 and ADAMTS-5 was not due to contaminating proteases present...
in our recombinant preparations, the study was repeated in the presence or absence of a mixture of protease inhibitors including E64 (cysteine proteases), phenylmethylsulfonyl fluoride (serine proteases), pepstatin A (aspartic proteases), and XS309 (matrix metalloproteinases) or in the presence of BB-16, which inhibits matrix metalloproteinases and ADAMTS-4 and ADAMTS-5. The mixture of protease inhibitors did not block conversion of intact \( \alpha_2 \text{M} \) by ADAMTS-4 (Fig. 3B) or ADAMTS-5 (data not shown) to the 98-kDa form. In contrast, BB-16, which is a potent aggreganase inhibitor, completely blocked conversion. These results suggest that cleavage of \( \alpha_2 \text{M} \) in these studies is mediated by ADAMTS-4 and ADAMTS-5 and not a contaminating protease. The higher molecular weight bands observed in lanes 2 and 3 reacted with a polyclonal antibody that recognizes the catalytic domain of ADAMTS-4 (35), confirming that these bands represent a covalent complex between activated \( \alpha_2 \text{M} \) and the proteinase (data not shown).

Truncated ADAMTS-4 (Phe213-Pro481) Cleaves \( \alpha_2 \text{M} \) — It has been shown that the thrombospondin motif of ADAMTS-4 is important for binding and cleavage of aggregan. In fact, a truncated form of ADAMTS-4 lacking the disintegrin and thrombospondin motifs located within the C-terminal portion of the molecule does not readily cleave native aggregan, even at concentrations as high as 200 nM, but maintains good activity against several peptide substrates (34). To determine whether the C-terminal regions of ADAMTS-4 are important for recognition and cleavage of \( \alpha_2 \text{M} \), the \( \alpha_2 \text{M} \) protein was incubated with truncated ADAMTS-4 (Phe213-Pro481) for 2 h at 37 °C. In the presence of truncated ADAMTS-4 complete conversion of intact \( \alpha_2 \text{M} \) to the 98-kDa species was observed, suggesting that the C-terminal domains of ADAMTS-4 are not required for cleavage of the macroglobulin molecule (Fig. 4).

ADAMTS-4 and ADAMTS-5 Cleave \( \alpha_2 \text{M} \) within the Bait Region at Met690 — To determine the site of cleavage(s) within \( \alpha_2 \text{M} \) the protein was incubated in the presence or absence of ADAMTS-4 or ADAMTS-5 for 6 h at 37 °C, and the products were analyzed by Coomassie stain. Analysis of the ADAMTS-4-\( \alpha_2 \text{M} \) and the ADAMTS-5-\( \alpha_2 \text{M} \) complex showed typical fragments of the \( \alpha_2 \text{M} \) subunits by SDS-PAGE (Fig. 5). N-terminal sequencing of the 98-kDa band, generated by both ADAMTS-4 and ADAMTS-5, revealed two N termini, 1SVSGKPQYMVLV and 691GRGHARLVHEEP, indicating that both ADAMTS-4 and ADAMTS-5 cleaved at the Met690/Gly691 bond (Fig. 5). This site of cleavage is not generated by other proteases inhibited by \( \alpha_2 \text{M} \), including collagenase, stromelysin, and trypsin and was found to be unique (41). Low levels of other N termini were detected, but the signal was too low to accurately identify (<0.2 pmol), indicating that ADAMTS-4 and ADAMTS-5 may cleave at additional sites within the bait region although much less efficiently than at the Met690/Gly691 bond.

Several peptides spanning the cleavage site Met690/Gly691 were synthesized (MGRG, VMGRGH, DVMGRGHA, SD-
The products were then separated by SDS-PAGE using an 8% gel and analyzed for total protein by Coomassie staining. B, 250 nM α2M incubated with 125 nM ADAMTS-4 in the presence or absence of a protease inhibitor mixture containing E64, phenylmethylsulfonyl fluoride, pepstatin A, and XSB-16 for 2 h at 37 °C. The products were separated on an 8% gel and analyzed for total protein by Coomassie staining. 1, α2M alone; 2, α2M + ADAMTS-4; 3, α2M + ADAMTS-4 + inhibitor mixture; 4, α2M + ADAMTS-4 + BB-16. MW, molecular weight.

Development of Neoepitope Antibodies That Recognize the New N Terminus (691GRGHAR) and the New C Terminus (YESDVM690) of α2M Fragments Generated upon Cleavage by ADAMTS-4 and -5—A polyclonal antibody to the new N terminus 691GRGHAR generated by cleavage of the α2M subunit at the Met690/Gly691 bond by ADAMTS-4 and ADAMTS-5 was developed, as described under “Experimental Procedures.” To test the ability of the antibody to detect ADAMTS-4/-5 generated α2M fragments, 50 ng of α2M was digested with 100 ng of ADAMTS-4 for varying periods of time at 37 °C. After the incubations, products were analyzed for 691GRGHAR macro- globulin fragments by SDS-PAGE using 4–12% gels followed by Western blot analysis. ADAMTS-4 generated a 98-kDa 691GRGHAR-containing fragment that increased in band intensity over time (Fig. 6A). This cleavage was a result of aggrecanase activity and not due to a contaminating proteinase in the enzyme preparations as both EDTA and the ADAMTS-4/-5 inhibitor, SC81956, blocked the generation of GRGHAR neo-epitopes by ADAMTS-4 (data not shown). To demonstrate specificity of the GRGHAR polyclonal antibody, the immunizing (GRGHAR) and spanning (YESDVMGRGHAR) peptides were analyzed for their ability to block antibody binding to ADAMTS-4-generated 691GRGHAR fragments. The immunizing peptide at a concentration of 10 μM completely blocked binding to the 98-kDa 691GRGHAR-containing fragment that increased in band intensity over time (Fig. 6A). This cleavage was a result of aggrecanase activity and not due to a contaminating proteinase in the enzyme preparations as both EDTA and the ADAMTS-4/-5 inhibitor, SC81956, blocked the generation of GRGHAR neo-epitopes by ADAMTS-4 (data not shown). To demonstrate specificity of the GRGHAR polyclonal antibody, the immunizing (GRGHAR) and spanning (YESDVMGRGHAR) peptides were analyzed for their ability to block antibody binding to ADAMTS-4-generated 691GRGHAR fragments. The immunizing peptide at a concentration of 1 μM completely blocked binding to the 98-kDa 691GRGHAR-containing fragment, whereas the spanning peptide did not block, confirming specificity of the antibody (Fig. 6A).

Similarly, a polyclonal antibody to the new C terminus YESDVMD690, also generated by cleavage of the α2M subunit at the Met690/Gly691 bond, was produced. As was seen with the GRGHAR antibody analysis of products of ADAMTS-4 digestion of α2M by Western blot demonstrated that ADAMTS-4 generated a ~98-kDa YESDVMD690-containing fragment that
increased in band intensity over time (Fig. 6B). Inclusion of inhibitors confirmed that generation of the YESDVM<sup>690</sup> neoepitope was the result of aggrecanase activity. Specificity of the YESDVM<sup>690</sup> polyclonal antibody was demonstrated by the ability of the immunizing (YESDVM) peptide to block and the inability of the spanning (YESDVMGRGHAR) peptide to block antibody binding to the 98-kDa YESDVM<sup>690</sup> fragment (Fig. 6B).

To demonstrate that cleavage of α<sub>2</sub>M within the bait region at Met<sup>690</sup> is not generated by other metalloproteinases, representative matrix metalloproteinases including MMP-3, MMP-13, and MMP-25 were analyzed for cleavage of α<sub>2</sub>M, and the products were analyzed by Western blot for all macroglobulin fragments generated using an anti-α<sub>2</sub>M polyclonal antibody and for macroglobulin fragments containing the N-terminal 691GRGHAR and C-terminal SDVM<sup>690</sup> by Western blot analysis using the 691GRGHAR (A) and YESDVM<sup>690</sup> (B) neoepitope antibody in the absence or presence of 1 μM immunizing or spanning peptide.

The 691GRGH Neoepitope Is Not Detected in OA Synovial Fluids—Fifteen synovial fluid samples from patients with radiographic and symptomatic knee OA were analyzed for the presence of α<sub>2</sub>M using the polyclonal antibody against α<sub>2</sub>M as well as ADAMTS-4/-5-generated fragments with the N-terminal 691GRGHAR antibody. OA synovial fluids contained mainly intact α<sub>2</sub>M migrating at a molecular mass of 180 kDa (Fig. 7). A small amount of cleaved α<sub>2</sub>M migrating at 98 kDa and a few very high and low molecular mass fragments were detected. The 691GRGH neoepitope could not be detected in any of the fluid samples. To exclude that this was due to the inability of ADAMTS-4 to cleave α<sub>2</sub>M in synovial fluid, the SF samples were incubated in the presence or absence of recombinant enzyme for 2 h at 37 °C. This resulted in the generation of a 98-kDa fragment and higher molecular weight fragments representing an SDS-stable complex between α<sub>2</sub>M and ADAMTS-4 (Fig. 7, A and B), demonstrating that ADAMTS-4 can cleave and, therefore, be inhibited by endogenous α<sub>2</sub>M in synovial fluid. Both the 98-kDa α<sub>2</sub>M fragment and the α<sub>2</sub>M-ADAMTS-4 SDS-stable complexes were de-
ADAMTS-4 and ADAMTS-5 cleave α₂-Macroglobulin

Fig. 7. Detection of the 691GRGH synovial fluid in OA synovial fluid. Synovial fluid was diluted 100-fold in buffer and incubated in the presence or absence of ADAMTS-4 at a concentration of 50 nM for 2 h at 37 °C. Cleavage of endogenous α₂M was measured by Western blot analysis using the α₂M polyclonal antibody (general cleavage) (A), 691GRGH neoptipe antibody (cleavage at Met690/Gly691) (B), and 691GRGH neoptipe antibody (C) in the presence of 1 μM immunizing peptide. 1, purified α₂M; 2, endogenous α₂M in the SF of a 49-year-old male; 3, endogenous α₂M in the SF of a 43-year-old male; 4, endogenous α₂M in the SF of a 62-year-old female; 5, endogenous α₂M in the SF of a 57-year-old male; 6, endogenous α₂M in the SF of a 52-year-old female.

Table I

| Aggrecanase cleavage sites | Reference |
|---------------------------|-----------|
| α₂M (Met690)              | FYESDRMGHRN |
| Agg (Glu773)              | RNTINEGAGS |
| Agg (Glu1545)             | STAISEGRTG |
| Agg (Glu1714)             | TTFKEELGSVE |
| Agg (Glu1819)             | QAPTAQEGERPS |
| Agg (Glu1919)             | EPTISPQGQRPP |
| Agg (Asn211)              | FDIPENRFVGG |
| Brev (Glu355)             | QEVESEEBRAIS |
| Ver (Glu451)              | KDPEAACEQRQY |
| ADAMTS4 (Glu753)          | DVRIPETATRNIK |
| ADAMTS4 (Thr811)          | PHEALTFFEQQ |
| ADAMTS4 (Lys694)          | GSGSFPEFRYDN |

Discussion

Both ADAMTS-4 and ADAMTS-5 are inhibited by α₂M. Inhibition of a protease by α₂M is initiated by proteolysis of the bait region of the macroglobulin molecule, which in turn triggers large conformational changes that entrap the protease. In the current studies we demonstrate that ADAMTS-4 and ADAMTS-5 cleave α₂M primarily between amino acids Met690 and Gly691. It is, therefore, concluded that in addition to serving as a potential endogenous inhibitor of ADAMTS-4 and ADAMTS-5, α₂M represents a substrate for both enzymes. The alignment of the aggrecanase cleavage sites known to date (Table I) indicates that glutamic acid at the P1 site and a small sequence around the aggrecanase cleavage site in aggrecan (Agg), brevican (Brev), versican (Ver), and (α₂M).

Importantly, the truncated form of ADAMTS-4, which is ineffective in cleaving native aggrecan, readily cleaves α₂M at the Met690/Gly691 bond. This raises the possibility that processing of aggrecanase proteins to remove the C-terminal disintegrin-like and thrombospondin motifs may result in a change in substrate specificity of these proteinases.

α₂M inhibits both ADAMTS-4 and ADAMTS-5 with second-order rate constants on the order of 10⁵ and 10⁴ M⁻¹ s⁻¹, respectively. These rates are intermediate between those reported for α₂M inhibition of human elastase (4.1 x 10⁷ M⁻¹ s⁻¹) and cathepsin G (3.7 x 10⁶ M⁻¹ s⁻¹), and those for kallikrein (4.8 x 10⁶ M⁻¹ s⁻¹), factor Xa (4.0 x 10⁴ M⁻¹ s⁻¹), and thrombin (4.9 x 10⁵ M⁻¹ s⁻¹) (37, 46). α₂M is found at very high concentrations in the synovial fluid higher than 2 mg/ml, suggesting that the half-lives of free ADAMTS-4 and ADAMTS-5 under these conditions would be on the order of 0.1 and 1 s, respectively. It is interesting to speculate that after cleavage of aggrecan, ADAMTS-4 and ADAMTS-5 bound to aggrecan fragments diffuse into the synovial fluid where they are entrapped by α₂M. The size of α₂M (725,000 daltons) precludes the enzymes from diffusing back into the cartilage extracellular matrix. If this is indeed the case it would suggest that α₂M may play a key role in determining the half-life of ADAMTS-4 and ADAMTS-5 in the articular joint.

In addition to α₂M it was recently shown that TIMP-3 is a potent endogenous inhibitor of the aggrecanases, with K(app)
values of 3.30 nM for ADAMTS-4 and 0.66 nM for ADAMTS-5 (19, 20). TIMP-3 was found to have a greater affinity for ADAMTS-4 and ADAMTS-5 than MMP-1, MMP-2, and MMP-3, suggesting that a primary physiological function for TIMP-3 may be the inhibition of the aggrecanases. Thus, both αM and TIMP-3 may be very important regulators of aggrecanase activity in the joint, and any factors causing an imbalance between these inhibitors and ADAMTS-4/ADAMTS-5 may result in the destruction of cartilage aggcan seen in arthritic diseases.

OA is a slowly progressing disease, and it can take many years before clinical symptoms are observed. Because of the slow progression of the disease, evaluation of disease-modifying osteoarthritis drug efficacy in clinical trials will be very difficult and costly. Therefore, the need to develop molecular biomarkers that can accurately monitor the progression and perhaps the onset of disease is critical. To this end potential biomarkers of OA have been identified that include both non-mechanism and mechanism-based biomarkers (47). However, these biochemical markers have yet to be validated. In the current studies we have identified these biochemical markers have yet to be validated. In the mechanism and mechanism-based biomarkers (47). However, these biochemical markers have yet to be validated. In the current studies we have identified these biochemical markers have yet to be validated.

ADAMTS-4 and ADAMTS-5 Cleave α2-Macroglobulin

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α2-Macroglobulin Is a Novel Substrate for ADAMTS-4 and ADAMTS-5 and Represents an Endogenous Inhibitor of These Enzymes

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