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α_2-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 aggrecan 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 α_2-macroglobulin (α_2M) as an additional endogenous inhibitor of ADAMTS-4 and ADAMTS-5. α_2M 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 10^6 and 10^5 M^{-1} s^{-1}, respectively. Inhibition of the aggrecanases was mediated by proteolysis of the bait region within α_2M, resulting in physical entrapment of these proteinases. Both ADAMTS-4 and ADAMTS-5 cleaved α_2M at Met^{690}/Gly^{691}, representing a novel proteinase cleavage site within α_2M and a novel site of cleavage for ADAMTS-4 and ADAMTS-5. Finally, the use of the anti-neoepitope antibodies to detect aggrecanase-generated α_2M-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).[1] 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 Glu^{373}, Glu^{1545}, Glu^{1714}, Glu^{1819}, and Glu^{1919} (human sequence) (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 Glu^{393} and Glu^{441}, 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 K_i(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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[1] The abbreviations used are: OA, osteoarthritis; MMP, matrix metalloproteinases; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; α_2M, α_2-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_2-Macroglobulin (α_2M), a general endoproteinase inhibitor, is a noncovalent tetramer of two 370-kDa disulfide-linked homodimers that circulates in blood at concentrations of ~2–4 mg/ml and is also found in the joint fluid at similar concentrations (25, 26). α_2M is active against most endoproteinases. Each subunit of the α_2M molecule contains a region referred to as the "bait region," a short stretch of amino acids (beginning at Pro^{667} and ending at Thr^{705}) 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 Cys^{949} and Glx^{952} in each subunit of α_2M (30). The activated thiol esters provide α_2M with a potential for covalent cross-linking of the activating proteinase through ϵ-lysyl (proteinase)-γ glutamyl-(α_2M) bonds and also for binding of other nucleophils present at activation (31, 32). α_2M 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 α_2M 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 Glu^{373}, forms an SDS-stable complex with α_2M (14, 33). Analysis of the 39 amino acids representing the bait region reveals 5 glutamic acids, Glu^{673}, Glu^{678}, Glu^{686}, Glu^{701}, and Glu^{702}, raising the possibility that ADAMTS-4/-5 may cleave α_2M. Therefore, the goals of the present studies were to determine 1) if α_2M can inhibit ADAMTS-4/-5 cleavage of aggrecan, 2) if inhibition is triggered by proteolysis of the bait region within the macroglobulin molecule, and 3) the aggrecanase site of cleavage within α_2M.

EXPERIMENTAL PROCEDURES

Materials—α_2M was purchased from Serva (Heidelberg, Germany). Full-length ADAMTS-4 and ADAMTS-5 were cloned and expressed in *Drosophila* Sf9 cells at Pfizer as previously described (6, 7). A truncated form of ADAMTS-4 beginning with residue Met^1, ending with residue Pro^{431}, and lacking the disintegrin and thrombospondin motifs was expressed at Dupont Pharmaceuticals in *Drosophila* cells, as described previously (34). The hydroxamic acids XS309 ([3*S*-[3*R**,2-[2*R**,2-(*R**,*S**)]-hexahydro-2-(2-[2-(hydroxyamino)-1-methyl-2-oxyethyl]-4-methyl-1-oxypentyl]-*N*-methyl]-3-pyridazinecarboxamide) and BB-16 (2*S*,2*R*,6*S*-3-aza-4-oxo-10-oxa-5-hexyl-2-(methylcarboxamido)-10-paracyclophane-6-*N*-hydroxycarboxamide) were synthesized at DuPont Pharmaceuticals. XS309 is a nanomolar inhibitor of a large number of MMPs but is inactive at <10 μM in blocking ADAMTS-4/ADAMTS-5. In contrast, BB-16 is a nanomolar inhibitor of both MMPs and ADAMTS-4/ADAMTS-5 (35). Compound SC81956 (s)-2-dimethylamino-*N*-hydroxy-3,3-dimethyl-4-[(4-phenoxyphenyl)sulfonyl]butanamide) was synthesized at Pfizer and is a potent nanomolar inhibitor of both ADAMTS-4 and ADAMTS-5. Methylamine and EDTA were purchased from Sigma. Polyvinylidene difluoride membranes, 8% and 4–12% gels were purchased from Invitrogen. Coomassie Brilliant Blue stain and destain were purchased from Bio-Rad. TIMP-3 was purchased from R&D Systems (Minneapolis, MN). A polyclonal antibody that recognizes native and denatured α_2M was purchased from RDI (Flanders, NJ). MMP-3 and MMP-13 were cloned and expressed at Pfizer. MMP-25 was a kind gift from Dr. Duanqing Pei (University of Minnesota). Human osteoarthritic synovial fluids were obtained from Dr. Kosei Ijiri (Orthopaedic Surgery Department, Kagoshima University, Japan). Samples were spun to remove cells and frozen at −80 °C for shipment to the United States.

Neoepitope Antibodies—Polyclonal neoepitope antibodies were prepared (Quality Controlled Biochemicals, Hopkinton, MA) to the peptide sequences YESDVM and GRGHAR that are present on the C terminus and N terminus of the α_2M subunit, respectively, generated by cleavage at the Met^{690}/Gly^{691} bond. A polyclonal neoepitope antibody to the peptide sequence AGEGPSGI that is present on the N terminus of aggrecan fragments generated by cleavage at the Glu^{1771}/Ala^{1772} bond was prepared as described previously (10). Intact undigested aggrecan was not detected by the anti-AGEGPSGI antibody. Only the immunizing peptide for the antibody blocked binding to the antigen, whereas the spanning peptide (TQAPTAQEAGEGPSGI) did not block binding, indicating that the antibody does not recognize the same sequence when present within uncleaved aggrecan.

Inhibition of ADAMTS-4/-5 by α_2M—45 μl of 2× buffer (100 mM Tris-HCl, 20 mM $CaCl_2$, and 200 mM NaCl, pH 7.5) were added to 25 μl of buffer (5 mM Tris, 10 mM NaCl, pH 7.5) containing ADAMTS-4 or -5 followed by 25 μl of buffer (5 mM Tris, 10 mM NaCl, pH 7.5) containing α_2M at a range of concentrations (concentrations based on the M_r of the tetramer). After incubation of samples for 2 h at 37 °C, 5 μl of aggrecan in water were added, and the samples were incubated for 30 min at 37 °C. The concentration of aggrecan was determined in the dimethylmethylene blue assay as previously described (36). Final concentrations in the reaction mixture were 25 nM ADAMTS-4 or ADAMTS-5, 500 nM aggrecan, and α_2M at 0, 5, 10, 15, 20, 27.5, 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 Glu^{1771} by Western blot analysis using the ^{1772}AGEG 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 AGEG product and quantifying the immunoreactive bands by scanning densitometry using the software OneDscan. Active site concentrations were determined by fitting these data to the equation $y = y_{max}(E_o - I_o)/(E_o - I_o\exp(k_i t(I_o - E_o)))$ (37), where y is the measured ^{1772}AGEG product, y_{max} is the amount of product in the absence of α_2M, k_i is the second-order rate constant, t is time, and E_o and I_o are the initial concentrations of enzyme and α_2M, respectively. For this purpose 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 10^5 and 10^4 M^{-1} s^{-1} for ADAMTS-4 and -5, respectively.

Time-dependent inhibition of ADAMTS-4/-5 by α_2M—45 μl of 2 × buffer (100 mM Tris-HCl, 20 mM $CaCl_2$, and 200 mM NaCl, pH 7.5) were added to 25 μl of buffer containing ADAMTS-4 at 2 nM or -5 at a concentration of 20 nM followed by 25 μl of buffer containing α_2M at 10 or 30 nM (ADAMTS-4 experiment) or α_2M at 100 or 300 nM (ADAMTS-5 experiment). After the incubation for various times at 37 °C, aggrecan was added to a final concentration of 500 nM in a volume of 5 μl, and samples were incubated for 30 min at 37 °C. The reactions were quenched by adding EDTA to a final concentration of 50 mM, and the products were analyzed for cleavage of aggrecan at Glu^{1771} by Western blot analysis using the ^{1772}AGEG neoepitope antibody. Control experiments showed no inhibition of ADAMTS-4 or -5 activity if α_2M and aggrecan were added at the same time without any preincubation. Thus, aggrecan appeared to be a completely effective inhibitor of α_2M action under these conditions.

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 α_2M and ADAMTS-4 and -5. The time course of inactivation at each concentration of enzyme and α_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 2.5 and 7.5 nM α_2M and 0.115 and 0.328 min^{-1} for 5 nM ADAMTS-5 in the presence of 25 and 75 nM α_2M, respectively. The linear dependence of inactivation rate on α_2M concentration suggested that at these concentrations it was proper to analyze this reaction as a simple second-order process. Dividing these observed rates by the α_2M 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, DYNAFIT (38) using the simple second-order model $E + I \rightarrow EI$ to yield estimates 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 $k_i = \ln(([E_0([I]_0 - [EI]))/([I]_0([E]_0 - [EI])))/(t([I]_0 - [E]_0))$, where k_i is the second-order rate constant, $[E]_0$ is the total enzyme concentration, $[I]_0$ is the total α_2M concentration, and [EI] is the concentration of enzyme-α_2M complex at time t (37).

Inactivation of α_2M by Methylamine—10 mg/ml α_2M was incubated with 100 mM sodium borate, pH 8.6, in the presence or absence of 400 mM methylamine for 2 h at 37 °C (39). Subsequently, the untreated and

methylamine-treated α_2M were tested for their ability to inhibit ADAMTS-4 and ADAMTS-5.

Cleavage of α_2M by ADAMTS-4/-5—ADAMTS-4 and ADAMTS-5 at a concentration of 200 or 112 nM, respectively, were incubated with 250 nM α_2M in buffer containing 50 mM Tris-HCl, 100 mM NaCl, and 10 mM $CaCl_2$, 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_2M by Truncated ADAMTS-4—250 nM α_2M was incubated with 200 mM mature truncated ADAMTS-4 (Phe^{213}-Pro^{431}) in buffer containing 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.5, for 2 h at 37 °C. After the digestions cleaved α_2M was detected by SDS-PAGE using an 8% gel followed by Coomassie staining. Truncated ADAMTS-4, lacking the C-terminal disintegrin-like and thrombospondin domains (Met^1-Pro^{431}), was expressed in *Drosophila* cells. When truncated or full-length ADAMTS-4 are expressed in these cells, the prodomain is removed by cleavage at Arg^{212}/Phe^{213} by a furin-like activity found in insect cells, resulting in the production of active enzyme. No latent form of the enzyme was detected in the conditioned media.

Aggrecan Isolation—Nasal septa were removed from bovine noses obtained fresh from the slaughterhouse (Covance, Denver, PA). The cartilage was sliced, and aggrecan was extracted by stirring at 4 °C for 48 h in 10 volumes of 4 M guanidinium-HCl in 0.05 M sodium acetate, pH 5.8, containing the protease inhibitors, 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 2 mM phenylmethanesulfonyl fluoride, and 0.05 M benzamidine HCl. Aggrecan monomers were isolated by equilibrium density gradient centrifugation in cesium chloride, and the bottom fraction of this gradient ($d > 1.54$ g/ml) containing aggrecan monomers was dialyzed at 4 °C against water, lyophilized, and stored at −20 °C.

Deglycosylation of Aggrecan Products—For analysis of fragments by Western blot aggrecan was enzymatically deglycosylated with chondroitinase ABC (0.1 unit/10 μg of aggrecan), keratanase (0.1 unit/10 μg of aggrecan), and keratanase II (0.002 unit/10 μg of aggrecan) for 3 h at 37 °C in buffer containing 50 mM sodium acetate, 0.1 M Tris/HCl, pH 6.5. After digestion the aggrecan was precipitated with 5 volumes of acetone and reconstituted in 30 μl of Tris-glycine SDS sample buffer containing 2.5% β-mercaptoethanol and heated for 10 min at 95 °C.

Western Blot Analysis—α_2M or aggrecan product for each sample was loaded on a 4–12% Tris-glycine gel and separated by SDS-PAGE under reducing conditions. The separated proteins were transferred to polyvinylidene difluoride membranes and immunolocated with a 1:1000 dilution of $YESDVM^{690}$ or ^{691}GRGHAR (for detecting α_2M) or ^{1772}AGEG (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.

N-terminal Sequencing—α_2M at a concentration of 1 μM was incubated with ADAMTS-4 and ADAMTS-5 at a concentration of 200 or 448 nM, respectively for 4 h at 37 °C. After the incubation, the products were separated by SDS-PAGE (8% acrylamide) and transferred to polyvinylidene difluoride membranes. The proteins were detected by staining with Coomassie Brilliant Blue R-250, and the bands of interest were excised, placed onto a polystyrene-treated glass filter, and sequenced in an Applied Biosystem 477A plus liquid sequencer with on-line 120A phenylthiohydantoin analysis.

Cleavage of α_2M Peptides—The following peptides were synthesized on an ABI solid phase peptide synthesizer using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protection chemistry: MGRG, VMGRGH, DVMGRGHA, SDVMGRGHAR, ESDVMGRGHAR, and YESDVMGRGHAR. 10 μM of each peptide (100 μl of total volume) in buffer containing 100 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35, pH 7.5, were incubated with ADAMTS-4 or ADAMTS-5 at a concentration of 50 nM. The digests were carried out for 18 h at 37 °C and analyzed by reverse phase high pressure liquid chromatography followed by electrospray mass spectrometry to verify cleavage and determine the site of cleavage from the mass of the fragments if generated.

Detection of the GRGH Neoepitope in Osteoarthritic Synovial Fluid—Synovial fluids were collected from patients with radiographic and symptomatic knee OA. Synovial fluids were diluted 100-fold in buffer containing 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, 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_2M at Met^{690}/Gly^{691} was monitored by Western blot analysis using the polyclonal antibody against α_2M or the ^{691}GRGHAR neoepitope antibody.

RESULTS

Inhibition of ADAMTS-4 and ADAMTS-5 by α_2M—We first examined the ability of α_2M 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_2M 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 generated by cleavage at the Glu^{1771}/Ala^{1772} bond by Western blot analysis using the ^{1772}AGEG neoepitope antibody. α_2M 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_2M (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_2M—ADAMTS-4 at a concentration of 0.5 nM was preincubated with α_2M 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 ^{1772}AGEG neoepitope antibody. Inhibition of ADAMTS-4 (Fig. 2) was concentration- and time-dependent. No inhibition of aggrecan cleavage at the Glu^{1771}/Ala^{1772} bond was observed when α_2M was not preincubated with ADAMTS-4, but after a 10-min preincubation ~100 and ~68% inhibition was achieved with α_2M 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_2M was examined (data not shown). A variety of approaches yielded estimates for the second-order rate constants for the reaction of α_2M with ADAMTS-4 and ADAMTS-5 to be on the order of 10^6 and 10^5 M^{-1} s^{-1}, respectively (see "Experimental Procedures").

ADAMTS-4 and ADAMTS-5 Cleave α_2M—α_2M inhibits most proteinases by physical entrapment upon cleavage within the bait region by the enzyme. This process is disturbed by the treatment of α_2M 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_2M failed to inhibit ADAMTS-4 and ADAMTS-5 (data not shown), suggesting that the inhibition of these two proteinases by α_2M is triggered by proteolysis of the bait region and not through allosteric binding and/or steric hindrance.

To confirm that α_2M is cleaved by ADAMTS-4 and ADAMTS-5, 250 nM α_2M 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_2M in its monomeric form was detected at a molecular mass of ~180 kDa. In the presence of ADAMTS-4 most of the intact α_2M was converted to a lower molecular mass species of ~98 kDa (Fig. 3*A*). In the presence of ADAMTS-5 about 50% of intact α_2M was converted to the 98-kDa species (Fig. 3*A*). These levels of conversion are consistent with 1:1 stoichiometry between inhibitor and enzyme. To confirm that cleavage of α_2M by ADAMTS-4 and ADAMTS-5 was not due to contaminating proteinases present

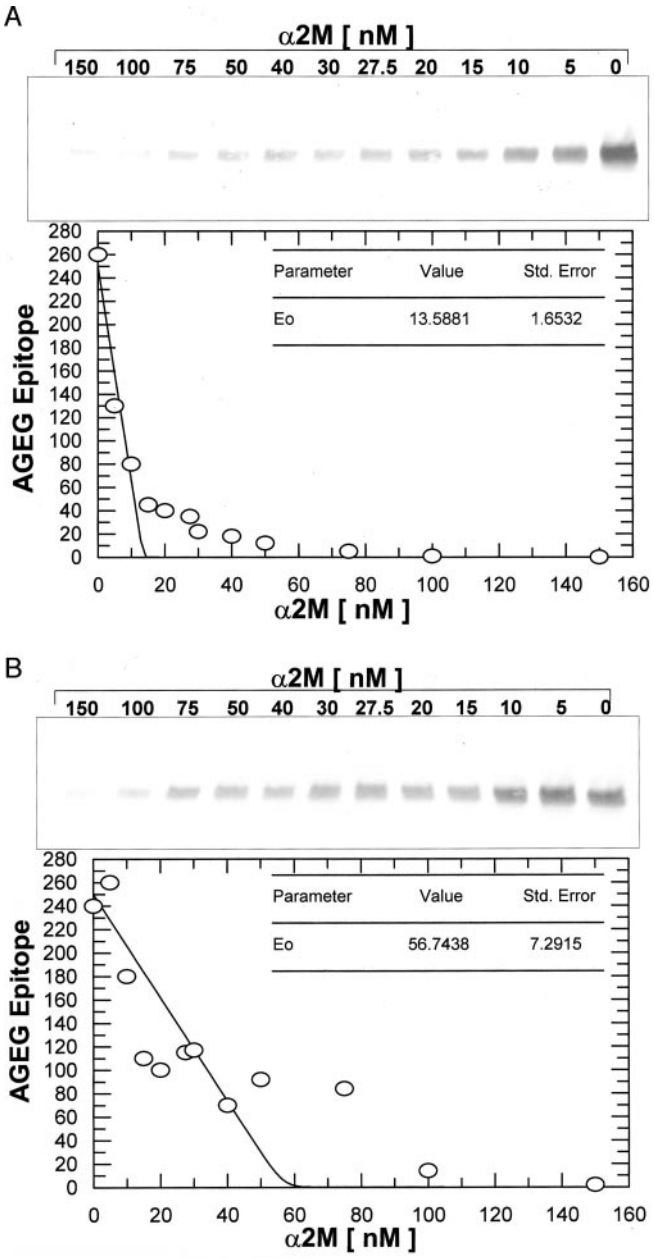

FIG. 1. **Inhibition of ADAMTS-4 and ADAMTS-5 by α_2M.** 25 nM ADAMTS-4 (*A*) or ADAMTS-5 (*B*) was incubated with α_2M at the following concentrations: 0, 5, 10, 15, 20, 27.5, 30, 40, 50, 75, 100, and 150 nM for 2 h at 37 °C. After the incubations aggrecan was added to a final concentration of 500 nM and allowed to incubate for 30 min at 37 °C. The reactions were subsequently quenched with 50 mM EDTA, and the products were analyzed for ^{1772}AGEG-reactive fragments by Western blot analysis using a ^{1772}AGEG polyclonal antibody. *Lines* indicate a best fit to an equation relating enzyme activity to α_2M concentration at a given time, enzyme concentration, and second order rate constant as described under "Experimental Procedures."

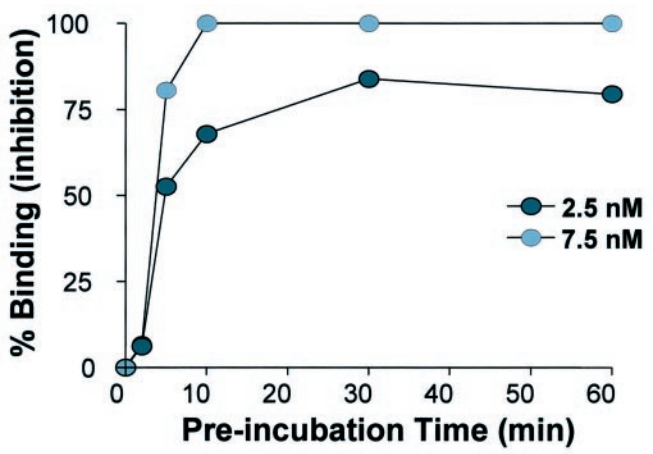

FIG. 2. **Inhibition of ADAMTS-4 by α_2M.** 0.5 nM ADAMTS-4 was incubated with α_2M at 2.5 or 7.5 nM for 0, 2, 5, 10, 30, and 60 min at 37 °C. After the incubations aggrecan was added to a final concentration of 500 nM and allowed to incubate for 30 min at 37 °C. After the incubations the reactions were quenched with 50 mM EDTA, and the products were analyzed for ^{1772}AGEG-reactive fragments by Western blot analysis using a ^{1772}AGEG neoepitope antibody. Blots were scanned, and the corresponding sum densitometry was plotted.

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 -5. The mixture of protease inhibitors did not block conversion of intact α_2M by ADAMTS-4 (Fig. 3*B*) or ADAMTS-5 (data not shown) to the 98-kDa form. In contrast, BB-16, which is a potent aggrecanase inhibitor, completely blocked conversion. These results suggest that cleavage of α_2M 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 α_2M and the proteinase (data not shown).

Truncated ADAMTS-4 (Phe^{213}-Pro^{481}) Cleaves α_2M—It has been shown that the thrombospondin motif of ADAMTS-4 is important for binding and cleavage of aggrecan. 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 aggrecan, 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 α_2M, the α_2M protein was incubated with truncated ADAMTS-4 (Phe^{213}-Pro^{481}) for 2 h at 37 °C. In the presence of truncated ADAMTS-4 complete conversion of intact α_2M 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 α_2M within the Bait Region at Met^{690}—To determine the site of cleavage(s) within α_2M 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-α_2M and the ADAMTS-5-α_2M complex showed typical fragments of the α_2M 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, ^{1}SVSGKPQYMVLV and ^{691}GRGHARLVHEEP, indicating that both ADAMTS-4 and ADAMTS-5 cleaved the subunit at the Met^{690}/Gly^{691} bond (Fig. 5). This site of cleavage is not generated by other proteases inhibited by α_2M, 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 Met^{690}/Gly^{691} bond.

Several peptides spanning the cleavage site Met^{690}/Gly^{691} were synthesized (MGRG, VMGRGH, DVMGRGHA, SD-

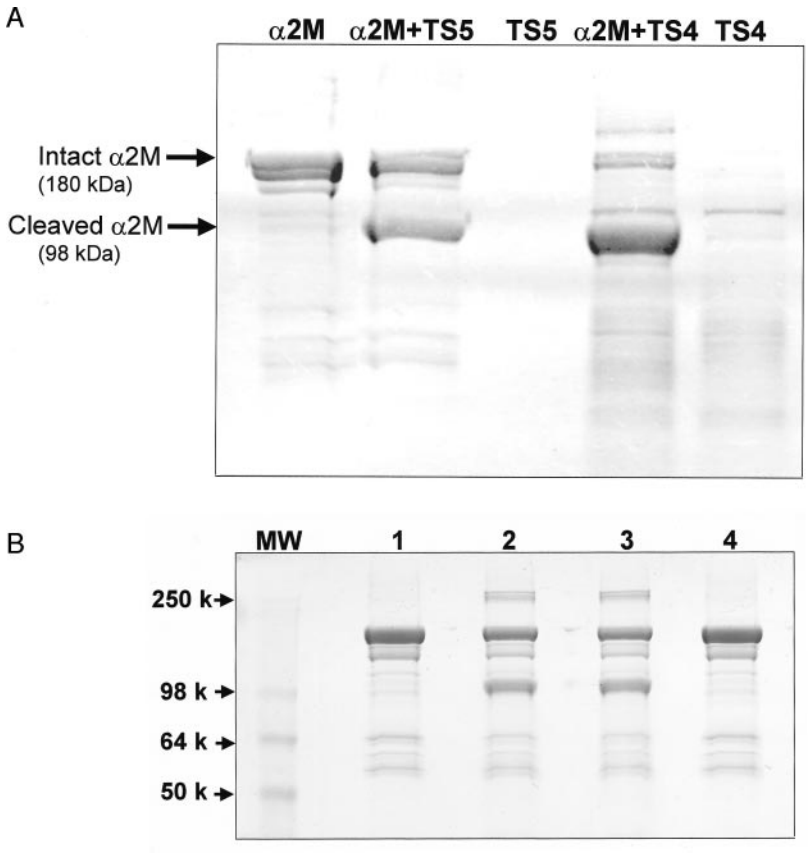

FIG. 3. **Cleavage of α_2M by ADAMTS-4 and ADAMTS-5.** *A*, 250 nM α_2M was incubated in the presence or absence of 200 nM ADAMTS-4 or 112 nM ADAMTS-5 for 2 h at 37 °C. The products were then separated by SDS-PAGE using an 8% gel and analyzed for total protein by Coomassie staining. *B*, 250 nM α_2M incubated with 125 nM ADAMTS-4 in the presence or absence of a protease inhibitor mixture containing E64, phenylmethylsulfonyl fluoride, pepstatin A, and XS309 or with BB-16 for 2 h at 37 °C. The products were separated on an 8% gel and analyzed for total protein by Coomassie staining. *1*, α_2M alone; *2*, α_2M + ADAMTS-4; *3*, α_2M + ADAMTS-4 + inhibitor mixture; *4*, α_2M + ADAMTS-4 + BB-16. MW, molecular weight.

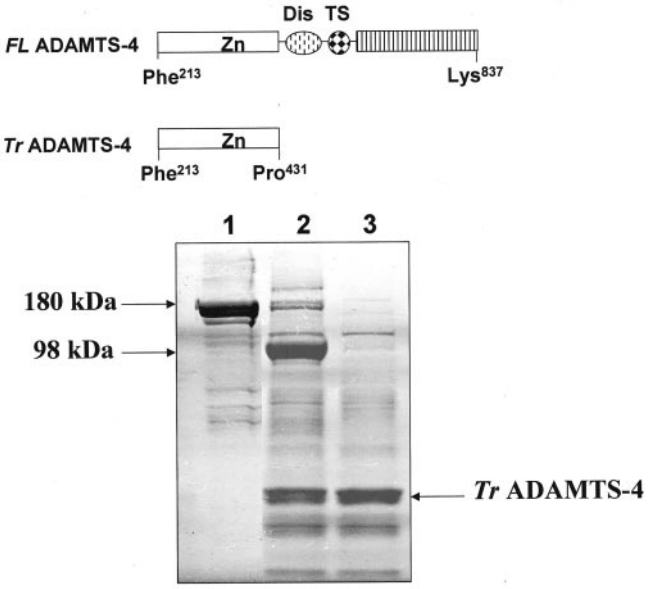

FIG. 4. **Cleavage of α_2M by truncated ADAMTS-4.** 250 nM α_2M was incubated in the presence or absence of 200 nM truncated ADAMTS-4 (Phe^{213}-Pro^{481}) for 2 h at 37 °C. The products were separated by SDS-PAGE using an 8% gel and analyzed for total protein by Coomassie staining. *1*, α_2M alone; *2*, α_2M + truncated ADAMTS-4; *3*, truncated ADAMTS-4 alone. *FL*, full-length; *Tr*, truncated.

VMGRGHAR, ESDVMGRGHAR, and SDVMGRGHAR) and analyzed for their ability to be hydrolyzed by ADAMTS-4 and ADAMTS-5. Surprisingly, none of the α_2M peptides was cleaved by ADAMTS-4 or ADAMTS-5, suggesting that in addition to the site of cleavage the local conformation around the bait region is required for hydrolysis (data not shown). This inability to cleave a peptide spanning an enzymatic cleavage site was not observed with trypsin, which is known to cleave α_2M after Arg as all peptides containing an internal Arg were cleaved by trypsin.

Development of Neoepitope Antibodies That Recognize the New N Terminus (^{691}GRGHAR) and the New C Terminus (YESDVM^{690}) of α_2M Fragments Generated upon Cleavage by ADAMTS-4 and -5—A polyclonal antibody to the new N terminus ^{691}GRGHAR generated by cleavage of the α_2M subunit at the Met^{690}/Gly^{691} 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 α_2M fragments, 50 ng of α_2M was digested with 100 ng of ADAMTS-4 for varying periods of time at 37 °C. After the incubations, products were analyzed for ^{691}GRGHAR macroglobulin fragments by SDS-PAGE using 4–12% gels followed by Western blot analysis. ADAMTS-4 generated a ~98-kDa ^{691}GRGHAR-containing fragment that increased in band intensity over time (Fig. 6*A*). 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 neoepitopes 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 ^{691}GRGHAR fragments. The immunizing peptide at a concentration of 1 μM completely blocked binding to the 98-kDa ^{691}GRGHAR fragment, whereas the spanning peptide did not block, confirming specificity of the antibody (Fig. 6*A*).

Similarly, a polyclonal antibody to the new C terminus YESDVM^{690}, also generated by cleavage of the α_2M subunit at the Met^{690}/Gly^{691} bond, was produced. As was seen with the GRGHAR antibody analysis of products of ADAMTS-4 digestion of α_2M by Western blot demonstrated that ADAMTS-4 generated a ~98-kDa YESDVM^{690}-containing fragment that

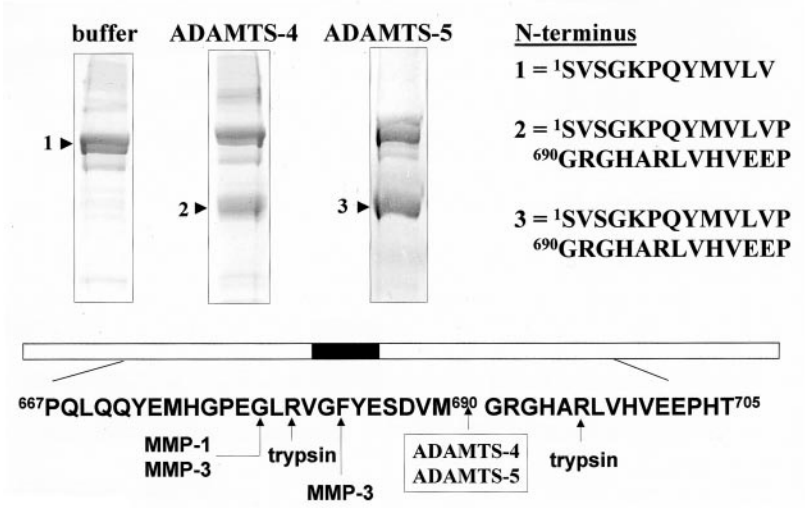

FIG. 5. **Aggrecanase site of cleavage within the bait region.** 1 μM α_2M was incubated in the presence or absence of 200 nM ADAMTS-4 or 448 nM ADAMTS-5 for 6 h at 37 °C. The products were separated on an 8% gel, transferred to polyvinylidene difluoride membranes, and analyzed for total protein by Coomassie stain. The N termini of each product were identified by Edman sequencing. *1*, intact α_2M; *2*, ADAMTS-4 digested α_2M; *3*, ADAMTS-5 digested α_2M.

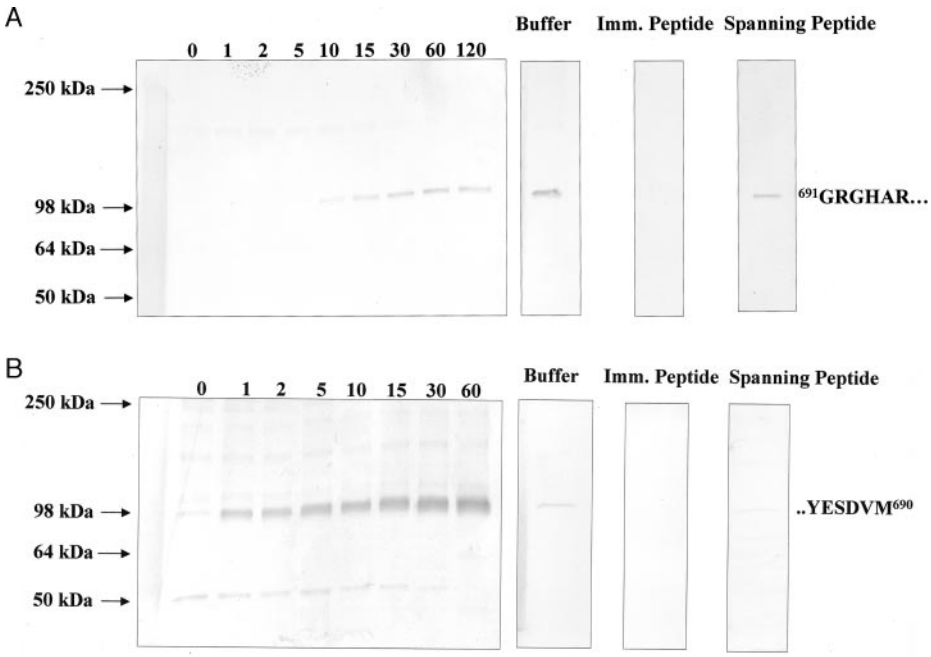

FIG. 6. **Detection of aggrecanase cleavage of α_2M at Met^{690}/Gly^{691} with a neoepitope antibody to ^{691}GRGHAR and to SDVM^{690}.** 50 ng of α_2M were digested with 100 ng of ADAMTS-4 at 37 °C for 0, 1, 2, 5, 10, 15, 30, 60, and 120 min. After the digestions the products were separated on a 4–12% gel and analyzed for macroglobulin fragments containing the N-terminal ^{691}GRGHAR and C-terminal SDVM^{690} by Western blot analysis using the ^{691}GRGHAR (*A*) and YESDVM^{690} (*B*) neoepitope antibody in the absence or presence of 1 μM immunizing or spanning peptide.

increased in band intensity over time (Fig. 6*B*). Inclusion of inhibitors confirmed that generation of the YESDVM^{690} neoepitope was the result of aggrecanase activity. Specificity of the YESDVM^{690} 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^{690} fragment (Fig. 6*B*).

To demonstrate that cleavage of α_2M within the bait region at Met^{690} is not generated by other metalloproteinases, representative matrix metalloproteinases including MMP-3, MMP-13, and MMP-25 were analyzed for cleavage of α_2M, and the products were analyzed by Western blot for all macroglobulin fragments generated using an anti-α_2M polyclonal antibody and for macroglobulin fragments generated by specific cleavage at the Met^{690}/Gly^{691} bond using the YESDVM^{690} and ^{691}GRGHAR neoepitope antibodies. ADAMTS-4, MMP-3, and MMP-13 cleaved intact α_2M, generating macroglobulin fragments with a molecular weight of ~98 kDa. MMP-25 under these conditions did not cleave α_2M. Unlike ADAMTS-4, neither MMP-3 nor MMP-13 generated macroglobulin fragments containing the ^{691}GRGHAR and YESDVM^{690} neoepitopes, suggesting that these enzymes do not cleave the bait region of α_2M at Met^{690} (data not shown).

The ^{691}GRGH 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_2M using the polyclonal antibody against α_2M as well as ADAMTS-4/-5-generated fragments with the N terminus ^{691}GRGHAR, using the neoepitope antibody. OA synovial fluids contained mainly intact α_2M migrating at a molecular mass of 180 kDa (Fig. 7). A small amount of cleaved α_2M migrating at 98 kDa and a few very high and low molecular mass fragments were detected. The ^{691}GRGH 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_2M 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_2M and ADAMTS-4 (Fig. 7, *A* and *B*), demonstrating that ADAMTS-4 can cleave and, therefore, be inhibited by endogenous α_2M in synovial fluid. Both the 98-kDa α_2M fragment and the α_2M·ADAMTS-4 SDS-stable complexes were de-

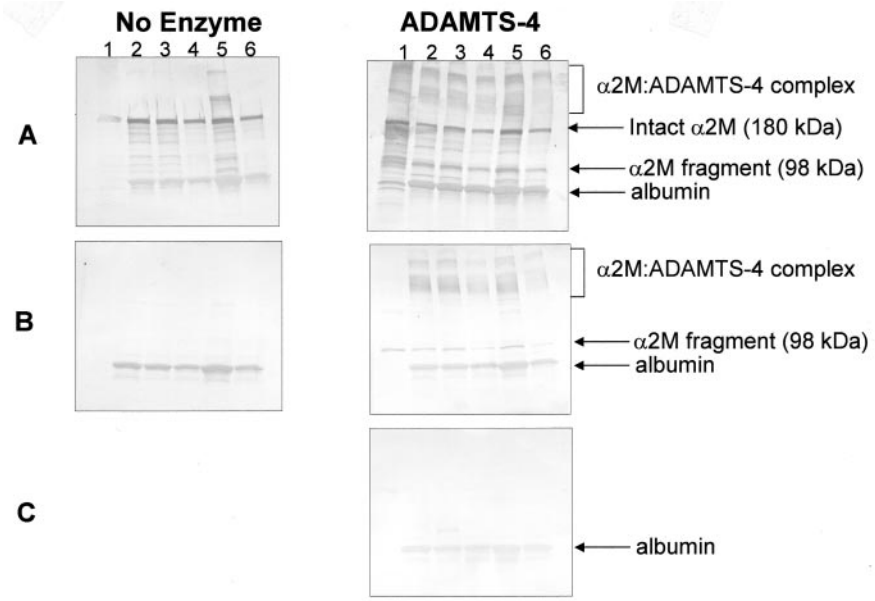

FIG. 7. **Detection of the ^{691}GRGH synovial fluid in OA synovial fluid.** Synovial fluid was diluted 100-fold in buffer and incubated in the presence or absence of ADAMTS-4 at a concentration of 50 nM for 2 h at 37 °C. Cleavage of endogenous α_2M was measured by Western blot analysis using the α_2M polyclonal antibody (general cleavage) (*A*), ^{691}GRGHAR neoepitope antibody (cleavage at Met^{690}/Gly^{691}) (*B*), and ^{691}GRGHAR neoepitope antibody (*C*) in the presence of 1 μM immunizing peptide. *1*, purified α_2M; *2*, endogenous α_2M in the SF of a 69 year-old male; *3*, endogenous α_2M in the SF of a 43-year-old male; *4*, endogenous α_2M in the SF of a 62-year-old female; *5*, endogenous α_2M in the SF of a 57-year-old male; *6*, endogenous α_2M in the SF of a 52-year-old female.

tected with the ^{691}GRGHAR neoepitope antibody, confirming cleavage of the α_2M subunit at the Met^{690}/Gly^{691} bond (Fig. 7, *A* and *B*). Blocking of these bands by the immunizing peptide confirmed specificity of the ^{691}GRGHAR neopitope antibody in detecting α_2M fragments and complexes with the N terminus ^{691}GRGHAR (Fig. 7*C*). Because of the high levels of albumin in synovial fluid, both the polyclonal antibody against α_2M and the ^{691}GRGH neoepitope antibody appeared to react with albumin. However, further analysis of this nonspecific binding revealed that it was due to the secondary antibody.

DISCUSSION

Both ADAMTS-4 and ADAMTS-5 are inhibited by α_2M. Inhibition of a proteinase by α_2M is initiated by proteolysis of the bait region of the macroglobulin molecule, which in turn triggers large conformational changes that entrap the proteinase. In the current studies we demonstrate that ADAMTS-4 and ADAMTS-5 cleave α_2M primarily between amino acids Met^{690} and Gly^{691}. It is, therefore, concluded that in addition to serving as a potential endogenous inhibitor of ADAMTS-4 and ADAMTS-5, α_2M 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 side chain at P1′ are favored residues. Surprisingly, ADAMTS-4 and ADAMTS-5 did not cleave (at detectable levels) at any of the five glutamic acid residues (Glu^{673}, Glu^{678}, Glu^{686}, Glu^{701}, and Glu^{702}) found within the bait region but, rather, cleaved preferentially at Met^{690}. The susceptibility of the Met^{690}/Gly^{691} bond in α_2M cannot be readily explained, but the sequence around the aggrecanase cleavage site in α_2M is similar to those found in the core protein of proteoglycans at P1′, P2′, and P3′ (GRG) (Table I). These data, along with recently published findings (42, 43) suggest that the aggrecanases are not exclusively glutamyl endopeptidases and that the presentation of the scissile bond along with the P′ amino acids may be more important than recognition of amino acids that have been speculated to be upstream from the scissile bond in aggrecan (44). Interestingly, neither ADAMTS-4 nor ADAMTS-5 cleaved any of the α_2M peptides spanning the aggrecanase cleavage site. These findings suggest that a combination of the sequence around the site of cleavage and the local conformation of the bait region is important for proteolytic cleavage at Met^{690}/Gly^{691}. This also suggests that ADAMTS-4 and ADAMTS-5 may not be limited to cleavage of aggregating proteoglycans but may cleave other proteins, as recently reported by others (43, 45). Importantly, the truncated form of ADAMTS-4, which is ineffective in cleaving native aggrecan, readily cleaves α_2M at the Met^{690}/Gly^{691} 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.

TABLE I
Aggrecanase cleavage sites

Alignment of the known aggrecanase cleavage sites for human aggrecan (Agg), brevican (Brev), versican (Ver), and (α_2M).

		Reference
α_2M (Met^{690})	FYESDV**MG**RGHAR	
Agg (Glu^{373})	RNITEG**E**ARGSVI	10
Agg (Glu^{1545})	STASEL**E**GRGTIG	10
Agg (Glu^{1714})	TTFKEE**E**GLGSVE	10
Agg (Glu^{1819})	QAPTAQ**E**AGEGPS	10
Agg (Glu^{1919})	EPTISQ**E**LGQRPP	10
Agg (Asn^{341})	FVDIPE**N**FFGVGG	42
Brev (Glu^{393})	QEAVES**E**SRGAIS	13
Ver (Glu^{441})	KDPEAA**E**ARRGQY	14
ADAMTS5 (Glu^{753})	DVVRIP**E**GATHIK	45
ADAMTS4 (Thr^{581})	PHGSAL**T**FREEQC	43
ADAMTS4 (Lys^{694})	GSGSFR**K**FRYGYN	43

α_2M inhibits both ADAMTS-4 and ADAMTS-5 with second-order rate constants on the order of 10^6 and 10^5 $M^{-1}s^{-1}$, respectively. These rates are intermediate between those reported for α_2M inhibition of human elastase (4.1×10^7 $M^{-1}s^{-1}$) and cathepsin G (3.7×10^6 $M^{-1}s^{-1}$), and those for kallikrein (4.8×10^4 $M^{-1}s^{-1}$), factor Xa (4.0×10^4 $M^{-1}s^{-1}$), and thrombin (4.9×10^2 $M^{-1}s^{-1}$) (37, 46). α_2M 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 α_2M. The size of α_2M (725,000 daltons) precludes the enzymes from diffusing back into the cartilage extracellular matrix. If this is indeed the case it would suggest that α_2M may play a key role in determining the half-life of ADAMTS-4 and ADAMTS-5 in the articular joint.

In addition to α_2M it was recently shown that TIMP-3 is a potent endogenous inhibitor of the aggrecanases, with K_i(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 α_2M 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 aggrecan 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 osteoarthritic 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 α_2M fragments containing the N-terminal ^{691}GRGHAR and the C-terminal YESDVM^{690} generated by cleavage of the α_2M subunit by ADAMTS-4/-5 as potential markers of aggrecanase activity. Cleavage of α_2M within the bait region at Met^{690} appears to be unique to the aggrecanases, as to date no other known proteinase has been shown to cleave at this site (48). However, the current methodology used failed to reveal these neoepitopes in OA synovial fluids. That we were unable to detect the α_2M·ADAMTS-4/-5 complex in OA synovial fluid suggests the complex is rapidly cleared through the vascular and lymph systems. It is well known that conformational change of α_2M leads to exposure of recognition sites that bind receptors such as the lipoprotein receptor-related protein on a variety of cells, including macrophages, resulting in clearance (49, 50). More sensitive techniques will have to be developed for detection in either the synovial fluid or the blood.

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