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ADAMTS-12 Associates with and Degrades Cartilage Oligomeric Matrix Protein*

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[3]The abbreviations used are:

OA osteoarthritis

COMP cartilage oligomeric matrix protein

ADAMTS a disintegrin and metalloproteinase with thrombospondin motifs

MMP matrix metalloproteinases

RA rheumatoid arthritis

RT-PCR reverse transcription polymerase chain reaction

TSP thrombospondin

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GST glutathione S-transferase

DBD DNA binding domain

SD synthetic dropout (base)

FOA fluoroorotic acid

CD catalytic domain

EGF epidermal growth factor

HA hemagglutinin

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Abstract

Loss of articular cartilage because of extracellular matrix breakdown is the hallmark of arthritis. Degradative fragments of cartilage oligomeric matrix protein (COMP), a prominent noncollagenous matrix component in articular cartilage, have been observed in the cartilage, synovial fluid, and serum of arthritis patients. The molecular mechanism of COMP degradation and the enzyme(s) responsible for it, however, remain largely unknown. ADAMTS-12 (a disintegrin and metalloprotease with thrombospondin motifs) was shown to associate with COMP both *in vitro* and *in vivo*. ADAMTS-12 selectively binds to only the epidermal growth factor-like repeat domain of COMP of the four functional domains tested. The four C-terminal TSP-1-like repeats of ADAMTS-12 are shown to be necessary and sufficient for its interaction with COMP. Recombinant ADAMTS-12 is capable of digesting COMP in vitro. The COMP-degrading activity of ADAMTS-12 requires the presence of Zn^{2+} and appropriate pH (7.5-9.5), and the level of ADAMTS-12 in the cartilage and synovium of patients with both osteoarthritis and rheumatoid arthritis is significantly higher than in normal cartilage and synovium. Together, these findings indicate that ADAMTS-12 is a new COMP-interacting and -degrading enzyme and thus may play an important role in the COMP degradation in the initiation and progression of arthritis.

More than 15% of the world population older than 18 years are affected by arthritic disorders, including osteoarthritis (OA)[3] and rheumatoid arthritis (RA) (1). Accumulating evidence suggests that proteases perform an important function in the breakdown of the extracellular matrix in OA and RA (2). Cartilage oligomeric matrix protein (COMP), a prominent noncollagenous component of cartilage, accounts for ~1% of the wet weight of articular tissue (3,4). COMP is a 524-kDa pentameric, disulfide-bonded, multidomain glycoprotein composed of approximately equal subunits (~110 kDa each) (5,6). Several studies suggest that monitoring of COMP levels (in both joint fluid and serum) can be used to assess the presence and progression of arthritis (7-11). Synovial fluid COMP levels were found to be higher in individuals with knee pain or injury (12), anterior cruciate ligament or meniscal injury (9,12), OA (8,12), and RA (7,13) than in healthy individuals.

Fragments of COMP have been detected in the cartilage, synovial fluid, and serum of patients with post-traumatic and primary OA and RA (7,8,13). The molecular mechanism of COMP degradation and the enzyme (s) responsible for it, however, remain largely unknown. Theoretically, inhibition of degradative enzymes can slow down or block the initiation and progression of arthritic diseases. The isolation of cartilage degradative enzymes is therefore of great interest from both a pathophysiological and a therapeutic standpoint. The ADAMTS family (ADAMTS: (a disintegrin and metalloprotease with thrombospondin motifs) consists of secreted zinc metalloproteinases with a precisely ordered modular organization that includes at least one thrombospondin type I repeat (14,15). Important functions have been established for several members of the ADAMTS family. ADAMTS-1, ADAMTS-4, ADAMTS-5, and ADAMTS-8 degrade the cartilage proteoglycan aggrecan and play a major role in aggrecan loss in arthritis (16-21). ADAMTS-5 was shown to be the major aggrecanase in mouse cartilage *in vivo* (22,23). ADAMTS-1 and ADAMTS-4 also participate in the turnover of the proteoglycans versican and brevican in blood vessels (24) and the nervous system, respectively

(25). ADAMTS-2, ADAMTS-3, and ADAMTS-14 are procollagen *N*-propeptidases (26,27). ADAMTS-2 mutations cause dermatosparaxis, an inherited disorder characterized by severe skin fragility (28). ADAMTS-13 is a von Willebrand factor-cleaving protease, and its mutations lead to heritable life threatening thrombocytopenic purpura (29-33). Several other ADAMTS enzymes whose functions are presently unknown, including ADAMTS-12, have been discovered through molecular cloning and classified as orphan ADAMTS. Expression profile analysis of the ADAMTS family in cartilage revealed that ADAMTS-12 is significantly higher in OA patients than in normal controls (1), indicating that ADAMTS-12 is probably an important enzyme that causes cartilage degradation in arthritic disorders.

In this study, we report the identification of ADAMTS-12 as a novel metalloproteinase known to bind to (through specific molecular domains for each binding partner) and degrade COMP. The relevance of this interaction is exemplified by the up-regulation of ADAMTS-12 mRNA in arthritic cartilage and synovium.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Yeast expression vectors pDBleu and pPC86 were obtained from Invitrogen. The segment encoding the four functional domains of mouse COMP: the N-terminal (amino acids 20-83), EGF repeat domain (amino acids 84-261), type III repeat domain (amino acids 266-520), and C-terminal domain (amino acids 521-755; GenBank™ accession number AF257516) were amplified by PCR and cloned in-frame into the SalI/NotI sites of pDBleu to generate pDB-COMP-NT, pDB-COMP-EGF, pDB-COMP-type III, and pDB-COMP-CT yeast expression constructs.

cDNA inserts encoding the following fragments of human ADAMTS-12 (See Fig. 4) were cloned in-frame into the SalI/NotI sites of pPC86 vector to generate the indicated plasmids: Fragment: Prodomain (amino acids 26-240), metalloproteinase domains (amino acids 241-463), disintegrin-like and cysteine-rich domain (amino acids 464-701), spacer-1 plus three middle TSP repeats (amino acids 702-995), spacer-2 plus C-terminal 4 TSP repeats plus C-terminal unique region (amino acids 996-1593), four C-terminal TSP repeats plus C-terminal unique region (amino acids 1316-1593), C-terminal unique region (amino acids 1531-1593), and four C-terminal TSP repeats (amino acids 1316-1530). Plasmid: pADAMTS-12 (26-240), pADAMTS-12-(241-463), pADAMTS-12-(464-701), pADAMTS-12-(702-995), pADAMTS-12-(996-1593), pADAMTS-12-(1316-1593), pADAMTS-12-(1531-1593), and pADA-MTS-12-(1316-1530).

The bacterial expression vector pGEX-3X (Invitrogen) was used to produce recombinant GST fusion proteins in *Escherichia coli*. The cDNA fragments encoding the catalytic domain of hADAMTS-12 (amino acids 241-463, GenBank™ accession number AJ250725) and the EGF-like domain of COMP (amino acids 84-261) were inserted in-frame into the BamHI/EcoRI sites of pGEX-3X to generate the pGEX12-CD and pGEX-COMP-EGF plasmids. The bacterial expression pBAD TOPO vector (Invitrogen) was used to produce His-tagged proteins in *E. coli*. A cDNA segment encoding the four C-terminal TSP motifs (His-TS12C4TSP) of hADAMTS-12 (amino acids 1316-1530) was subcloned into the pBAD TOPO vector per the manufacturer's protocol. All constructs were verified by nucleic acid sequencing; subsequent analysis was performed using Curatools (Curagen, New Haven, CT) and BLAST software.

Expression and Purification of GST and His-tagged Proteins—For expression of GST fusion proteins, the pGEX12-CD and pGEX-COMP-EGF plasmids were transformed into *E. coli* DH5α (Invitrogen). Fusion proteins were affinity-purified on glutathione-agarose beads as previously described (34). To cleave off and remove the GST moiety from the GST fused catalytic domain of ADAMTS-12, 50 μg of purified GST-TS12-CD fusion protein was

incubated with 1 μg of Xa factor (New England Biolabs, Beverly, MA) in 20 μl of 20mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM $CaCl_2$ at 23 °C for 8 h. The reaction was terminated by the addition of 2 μM dansyl-Glu-Gly-Arg-chloromethyl ketone (New England Biolabs) and incubated at room temperature for 1 min. The completion of the cleavage was established by SDS-PAGE and the resultant GST moiety was removed using glutathione-Sepharose-4B beads (Amersham Biosciences).

His-TS12C4TSP was purified by affinity chromatography using a HiTrap chelating column (Amersham Biosciences). Briefly, bacteria lysates supplemented with 20 mM HEPES, pH 7.5 and 0.5 M NaCl were applied to the HiTrap chelating column, the column was washed with HSB buffer (40 mM HEPES, pH 7.5, 1 M NaCl, 0.05% Brij 35) containing 10 mM imidazole, and the His-TS7C4TSP was eluted with HSB buffer containing 300 mM imidazole.

Assay of Protein-Protein Interactions using the Yeast Two-hybrid System—Three independent colonies were analyzed for interaction in yeast of two proteins, one of which was fused to the Gal4 DNA binding domain and the other to the VP16 transactivation domain. The procedures of Vojtek *et al.* (35) and Hollenberg *et al.* (36) were followed for (*a*) growing and transforming the yeast strain MAV203 with the selected plasmids; and (*b*) testing β-galactosidase activity and growth phenotypes on (S.D.-leu^-/trp^-/his^-/ura^-/$3AT^+$) plates and on plates containing 5-fluoroorotic acid (S.D.-leu^-/trp^-/$5FOA^+$).

In Vitro GST Pulldown Assay—To determine whether COMP binds to ADAMTS-12 *in vitro*, glutathione-Sepharose beads (50 μl) preincubated with either purified GST (0.5 μg; serving as control) or GST-COMP-EGF (0.5 μg) were incubated with 500 μg of cell lysates prepared from COS-7 cells transfected with an expression plasmid either wild-type ADAMTS-12 (pcDNA3-ADAMTS12-HA) or ADAMTS-12 with mutant catalytic domain (pcDNA3-ADAMTS12-MUT, provided by Drs. Cal and Lopez-Otin) (39) in 150 μl of buffer AM (10 mM Tris-HCl, pH. 7.9, 10% glycerol, 100 mM KCl, and 0.5 mg/ml bovine serum albumin). The bound proteins were denatured in sample buffer, resolved by 10% SDS-PAGE, and detected by Western blotting with anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Solid Phase Binding Assay—Microtiter plates (96-well EIA/RIA plates, Costar, Badhoevedorp, The Netherlands) were coated with various amounts (0.001-5.000 μg) of purified His-TS12C4TSP in 100 μl of TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) overnight at 4 °C. Wells were blocked with 1% bovine serum albumin in TBS buffer for 3 h at 37 °C. After washing with TBS and 0.05% Tween, 100 μl of 50 μg/ml of COMP was added to each well, followed by the addition of 10 mM $CaCl_2$; samples were then allowed to bind overnight at 4 °C. Bound protein from the liquid phase was detected by mouse monoclonal antibody against COMP, followed by a secondary antimouse antibody conjugated with horseradish peroxidase (Antigenix America, Huntington Station, NY) and 5-amino-2-hydroxybenzoic acid as a substrate, with absorbance measured at 492 nm in an ELISA reader.

Coimmunoprecipitation—COMP stable line was transfected with a mammalian expression construct pcDNA3-ADAMTS12-HA that encodes a HA-tagged ADAMTS-12 (generously provided by Drs. S. Cal and C. Lopez-Otin) (39). 48 h after transfection, the cultures and media were extracted with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4 containing the proteinase inhibitors 1 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide, and 0.025 mg/ml leupeptin). Approximately 500 μg of cell extract was incubated with anti-COMP (25 μg/ml), anti-HA (25 μg/ml, Santa Cruz Biotechnology) or control rabbit IgG (25 μg/ml) antibodies for 1 h, followed by incubation with 30 μl of protein A-agarose (Invitrogen) at 4 °C overnight. After washing five times with immunoprecipitation buffer, bound proteins were released by boiling in 20 μl of 2× SDS loading buffer for 3 min (40). Released proteins were

examined by Western blotting with anti-COMP antibodies, and the signal detected using the ECL chemiluminescent system (Amersham Biosciences).

In Vitro Digestion Assay—To determine whether ADAMTS-12 can digest COMP *in vitro*, a cotransfection assay was performed. Briefly, a COMP stable line was transfected with either control or expression constructs encoding wild-type ADAMTS-12 (pcDNA3-ADAMTS12-HA-FLAG) or ADAMTS-12 with mutant catalytic domain (pcDNA3-ADAMTS12-MUT) (39). Seventy-two hours after transfection, the media were collected and subjected to 8% nonreduced SDS-PAGE and intact COMP, and fragments were detected by Western blotting with polyclonal rabbit anti-COMP antiserum, as previously described (13,37,38). An *in vitro* digestion assay with purified COMP and the cell lysates carrying ADAMTS-12 was then performed. Briefly, purified COMP (200 nM) was incubated with the cell lysates and medium prepared from COS-7 or HEK293 cells transfected with the above-mentioned plasmids in a digestion buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$,2mM $ZnCl_2$ 0.05% Brij-35, pH 7.5) at 37 °C for 12 h. The digested products were analyzed as described above. Finally, we examined the degradation of COMP mediated by purified ADAMTS-12. Briefly, medium was collected from COS-7 or HEK293 cells transfected with the above-mentioned plasmids and 50 μl of anti-FLAG M2 antibody (Sigma) was added to the medium followed by 50 μl of protein A-agarose. After washing, purified ADAMTS-12 was incubated with purified COMP and the digestion performed as described above.

To determine whether the enzymatic activity of ADAMTS-12 depends on divalent cations, purified COMP substrate (250 nM)was incubated with purified catalytic domain of ADAMTS-12 (25 nM)in digestion buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) supplemented with 5 mM $CaCl_2$,2mM $ZnCL_2$, 2.5 mM $MgCl_2$,5mM EDTA or various combinations at 37 °C for 12 h. The digested products were resolved by 10% nonreduced SDS-PAGE, and the gel were stained with Coomassie Brilliant Blue G-Colloidal solution.

To test the enzymatic activity of ADAMTS-12 in the presence of different amount of Zn^{2+} or at different pH values, the same digestion was performed in a buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$) containing various concentration of Zn^{2+} (0, 0.5 mM, 1.0 mM, 2.0 mM, 4.0 mM 8.0 mM) or in a buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 2.0 mM Zn Cl_2, 2.5 mM $MgCl_2$) at various pH values (4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5) at 37 °C for 12 h, and digested proteins were processed as above.

RNA Preparation and Reverse Transcription PCR—Human meniscus, bone, cartilage, synovium, ligament, tendon, fat, and skeletal muscle obtained from four normal human knees (provided by the Musculoskeletal Transplant Foundation, Edison, NJ), were frozen immediately after isolation and ground under liquid nitrogen (41). Total RNA was extracted by the acid-guanidium thiocyanate-phenol-chloroform single-step method followed by RNAeasy kit (Qiagen, Valencia, CA). One microgram of total RNA per sample was reverse-transcribed using the ImProm-II Reverse Transcription system (Promega). The following sequence-specific primers were synthesized: 5′-GTGGAACGGGAAC-TATAAGCTG-3′ and 5′-GTTTCAGAACTCTCCGGCTAGA-3′ for human ADAMTS-12. The following pair of oligonucleotides was used as internal controls: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ for human GAPDH.

PCR was performed for 35 cycles (94 °C 1 min, 60 °C 1 min, and 72 °C 1 min) with a final elongation for 10 min at 72 °C. GAPDH was also amplified as an internal control for 35 cycles (94 °C 1 min, 55 °C 1 min, 72 °C 1.5 min). The PCR product was analyzed by 1% agarose gel electrophoresis and further sequenced by the Applied Biosystems sequencing system (Foster City, CA).

Expression of ADAMTS-12 in Arthritic Tissues Assayed by TaqMan Real Time PCR—Normal adult articular cartilage and synovium were obtained from the knees of four patients (mean age 56.7 years, range 43-64 years) who had died of diseases unrelated to arthritis (specimens obtained en bloc from the Musculoskeletal Transplant Foundation). The grade of osteoarthritis was determined using the Kellgren-Lawrence Grading System (42). Normal tissues samples were without radiographic or intraarticular evidence of arthritic disease (Kellgren-Lawrence, Grade 0). Arthritic cartilage and synovium were obtained from 12 patients undergoing elective total knee arthroplasty for end-stage arthritis: OA articular cartilage (Kellgren-Lawrence, Grade 3 or 4) from the distal femora of 8 patients (mean age: 58.4 years, range 49-66 years) and RA cartilage (American College of Rheumatology Stage III and IV disease) and synovium from the knees of 4 RA patients (mean age: 57.8, range 45-67) who fulfilled the revised criteria of the American College of Rheumatology for the diagnosis of RA (43).

Following total RNA extraction and reverse transcription, real-time PCR was performed using a sequence-specific probe and primers for ADAMTS-12 (fluorescence-labeled oligonucleotide probes (using 6-carboxy-fluorescein (FAM)) probe: AGGACATCTGTGCTGGTTT-CAATCGCC*;* primers: CACGACGTGGCTGTCCTTCT and CCGAA-TCTTCATTGATGTTACAACTG). The correction of the PCR products obtained was confirmed by direct sequencing of the amplicons. A standard curve with copy numbers ranging from 10^3 to 10^9 was produced using human cartilage cDNA as the template. An *XY* scatter plot was produced using Microsoft Excel software, and the equation $y = mx + b$ (where m = the slope of the standard curve and b = the y-intercept of that line) was calculated and R^2 values obtained. As an internal control, 18 S rRNA was analyzed in parallel by using the Endogenous Control Human rRNA kit (Applied Biosystems).

PCR reactions for all samples were performed in duplicate in 96-well optical plates with 5 ng of cDNA (1 ng of cDNA for the 18 S rRNA), 100 nM probe, 200 nM each primer, and 10.0 μl of TaqMan Universal 2× PCR Master Mix (PE-Applied Biosystems, St. Louis, MO) in a 20-μl reaction volume. The amplification reaction was carried out over 40 cycles (an initial holding stage of 2 min at 50 °C and then 10 min at 95 °C, followed by a two-step cycling program of 15 s at 95 °C and 1 min at 60 °C).

RESULTS

ADAMTS-12 Associates with COMP in Yeast—Our unpublished observation that the EGF-like domain of COMP binds to the C-terminal TSP1-like repeats of ADAMTS-7, whose domain organization and structure are similar to those of ADAMTS-12, prompted us to investigate whether ADAMTS-12 interacts with COMP. For this purpose, the four C-terminal TSP1-like repeats of ADAMTS-12 (amino acids 1316-1530) were subcloned into a yeast expression pPC86 vector and a yeast two-hybrid assay performed (Fig. 1A). Briefly, the plasmid encoding the EGF-like domain of COMP (amino acids 84-261) linked to Gal4DBD (above the line in Fig. 1B) and the plasmid encoding the four C-terminal TSP1-like repeats of ADAMTS-12 fused to VP16AD (below the line) were used to cotransform the yeast strain MAV203. Plasmid pairs encoding c-Jun/c-Fos and Rb/lamin were used as positive and negative protein-protein interaction controls, respectively. Interaction between the C-terminal polypeptide of ADAMTS-12 and the EGF-like domain of COMP was resolved by a β-galactosidase assay and growth phenotype on selective media (Fig. 1B). Like the c-Jun/c-Fos pair, which are known to interact, the EGF-like domain of COMP was shown to interact with the C-terminal of ADAMTS-12 in yeast based on β-galactosidase activity (Fig. 1B, left) and growth inhibition on plates containing 5-fluoroorotic acid (two hybrid-dependent activation of URA3 results in conversion of 5-fluoroorotic acid to 5-fluorouracil, which is toxic). Hence, the growth of yeast containing interacting proteins is inhibited when plated on the medium containing 5-fluoroorotic acid (Fig. 1B, right).

Direct Binding of COMP to the C-terminal Polypeptide of ADAMTS-12 in Vitro—The interaction between COMP and ADAMTS-12 was also confirmed using *in vitro* GST pulldown assays. Briefly, affinity-purified GST and a purified COMP EGF-like domain (amino acids 84-261) as a GST fusion protein (GST-EGF) that were immobilized on glutathione-Sepharose beads were incubated with cell extracts prepared from COS-7 cells transfected with an expression plasmid encoding either wild-type ADAMTS-12 (TS-12, Fig. 2A) or ADAMTS-12 with mutant catalytic domain (TS-12mut, Fig. 2B) (39). After washing, glutathione-Sepharose bead bound proteins were resolved by 10% SDS-PAGE and Western blotting. Purified GST did not pull down either wild-type or mutant ADAMTS-12 (Fig. 2A, lane 2 and B, lane 2), whereas GST-EGF efficiently pulled down these proteins (Fig. 2A, *lane 3* and *B, lane 3*), indicating that the EGF-like domain of COMP binds to the ADAMTS-12 *in vitro*.

The interaction between COMP and ADAMTS-12 was also characterized by an *in vitro* solid-liquid phase titration experiment in which the dilution series of recombinant His-TS12C4TSP and purified COMP showed dose-dependent binding and saturation to the liquid-phase COMP (Fig. 2C). The interaction between COMP and ADAMTS-12 is direct, since both COMP and the C-terminal four TSP-1-like repeats were used as purified recombinant proteins.

Binding of COMP to ADAMTS-12 in Mammalian Cells—We next performed a coimmunoprecipitation assay to determine whether ADAMTS-12 associates with COMP *in vivo* (Fig. 2D). COMP stable cell lines were transfected with a mammalian expression pcDNA3-ADAMTS12-HA plasmid that encodes HA-tagged ADAMTS-12 (provided by Drs. Cal and Lopez-Otin) (39). The cell extracts prepared from those transfected cells were first incubated with control IgG (negative control), anti-COMP (positive control), or anti-HA, and the complexes were detected with anti-COMP polyclonal antiserum. A specific COMP band was immunoprecipitated by anti-COMP antibodies (lane 3) and anti-HA antibodies (lane 4), but not by control IgG antibodies (lane 2), demonstrating that ADAMTS-12 specifically associates with COMP *in vivo*.

Selective Association of ADAMTS-12 with the EGF-like Domain of COMP—As shown in Fig. 1, ADAMTS-12 associates with the EGF-like domain of COMP. We also investigated whether ADAMTS-12 interacts with the other three functional domains of COMP, including the N-terminal pentamerizing domain, the type III domain, and the C-terminal global domain. For this purpose, filter-based β-galactosidase assays were performed to determine whether coexpression of the various domains of COMP/Gal4DBD and ADAMTS-12/VP16AD fusion proteins activate the reporter LacZ gene. As shown in Fig. 3, of the four functional domains tested, ADAMTS-12 selectively interacts with only the EGF-like domain of COMP.

Four C-terminal TSP1-like Repeats of ADAMTS-12 Are Necessary and Sufficient for Binding COMP—To identify the COMP binding domain in ADAMTS-12, we generated various constructs that expressed various ADAMTS-12 deletion mutants in yeast. Results from filter-based β-galactosidase assays (Fig. 4B) of all these mutants are summarized in Fig. 4A. The ADAMTS-12 prodomain, the metalloproteinase, disintegrin-like, and cysteine-rich domains, and the spacer-1 plus three TSP repeats all failed to bind to COMP. As expected, the spacer-2 plus four C-terminal TSP-1-like repeats bound to COMP. When the spacer-2 domain was removed, the binding to COMP was not disturbed, indicating that this domain is not required for binding. Further removal of four TSP-1-like repeats eliminated binding, indicating that four TSP-1-like repeats in the C-terminal are required. When four TSP-1-like repeats were used, however, COMP binding occurred. Our conclusion from this set of experiments is that that four C-terminal TSP-1-like repeats of ADAMTS-12 are required and sufficient for its interaction with COMP.

Cleavage of COMP by Recombinant ADAMTS-12 in Vitro—Once the interaction between ADAMTS-12 and COMP was established, cotransfection assays were performed to determine whether ADAMTS-12 has COMP-degrading activity. A COMP stable line was transfected with empty pcDNA3 vector, wild-type ADAMTS-12 (pFLAG-TS-12), or ADAMTS-12 with mutant catalytic domain (pTS-12mut), and the medium collected and detected by Western blotting with anti-COMP antibodies (13,37,38). As shown in Fig. 5A, the COMP stable cells transfected with either empty vector (lane 3) or mutant ADAMTS-12 (in which two crucial amino acids in the catalytic domain of ADAMTS-12 were mutated) (39) (lane 2) did not show any COMP-degrading activity, but wild-type ADAMTS-12 (lane 1) digested COMP and produced one major fragment with an apparent molecular mass of ~100 kDa (large arrowhead) and two minor fragments (small arrow-heads). We verified the COMP-cleaving activity of ADAMTS-12 by an *in vitro* digestion assay with purified COMP and the cell lysates expressing ADAMTS-12 or its mutant. As indicated in Fig. 5B, cell extracts transfected with an empty vector (lane 1) did not result in any COMP-degrading activity, but wild-type ADAMTS-12 (lane 2) showed COMP-degrading activity. In addition, enzymatic activity was dramatically reduced when the two crucial amino acids in the catalytic domain of ADAMTS-12 were mutated (39) (lane 3). COMP-degrading activity by ADAMTS-12 was further demonstrated by digestion performed with purified ADAMTS-12 (Fig. 5C).

Cleavage of COMP by ADAMTS-12 Is Zn^{2+}- and pH-dependent—It was reported that the catalytic domain of ADAMTS-20 produced in bacteria can digest its substrates *in vitro* (44). Using a similar method, we purified the catalytic domain (amino acids 241-464) of ADAMTS-12 as a GST fusion protein (GST-TS12-CD) in bacteria. The GST moiety was further removed by a Xa factor, and the purity of proteins was confirmed by silver staining (not shown). The recombinant catalytic domain of ADAMTS-12 was employed in the following *in vitro* digestion assays. To determine whether ADAMTS-12-mediated COMP cleavage requires the involvement of cations, including Ca^{2+}, Zn^{2+}, and/or Mg^{2+}, purified COMP substrate and ADAMTS-12 enzyme were incubated in digestion buffer in the presence or absence of various cations (Fig. 5D). A degraded COMP fragment (*arrow* in Fig. 5D) was detectable in the digestion buffer with Zn^{2+} (lane 6) but was undetectable in the digestion buffer with Ca^{2+} (lane 5) or Mg^{2+} (lane 7) used alone. In the presence of Zn^{2+} (compare lanes 8 and 10), the addition of Ca^{2+} changed the electrophoretic mobility of the COMP-digested fragment, probably due to a conformation change in COMP. Mg^{2+} seems not to affect COMP digestion by ADAMTS-12, since it did not affect the electrophoretic mobility of the COMP-digested fragment when used alone or in combination with other cations (compare lanes 10 and 7).

Because Zn^{2+} is essential for the enzymatic activity of ADAMTS-12, we next performed an *in vitro* digestion assay with different Zn^{2+} concentrations (0, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, and 8.0 m_M) to determine the optimum concentration of Zn^{2+} for ADAMTS-12 activity (Fig. 5E). The highest enzymatic activity was observed in the presence of 2 m_M of $ZnCl_2$, and the amount of digested COMP was dramatically reduced or totally abolished in the presence of higher amounts of $ZnCl_2$ (4 or 8 m_M).

In vitro digestions were performed at various pH values in order to examine its regulation of ADAMTS-12 activity. As shown in Fig. 5F, ADAMTS-12 generated the largest amount of COMP fragments in the range of physiological pH (pH 7.5) up to pH 9.5, whereas the enzyme did not produce visible COMP fragments at pH values lower than 6.5 or higher than 10.5, indicating that the digestive activity of ADAMTS-12 is pH-dependent.

Musculoskeletal Tissues Distribution of ADAMTS-12—The fact that COMP is predominately expressed in the musculoskeletal tissues, together with our findings that ADAMTS-12 binds to and cleave COMP, prompted us to test the expression of ADAMTS-12 in musculoskeletal tissues. Reverse-transcription PCR (RT-PCR) assay was performed to examine the expression

of ADAMTS-12 mRNA in eight specimens of normal human musculoskeletal tissue. As seen in Fig. 6A, the 733-bp hADAMTS-12 fragment was amplified using ADAMTS-12-specific primers from cartilage, synovium, and tendon in which COMP was also present (3,4). ADAMTS-12 is also detectable in skeletal muscle and fat. However, ADAMTS-12 was undetectable in meniscus, bone, and ligament. These results demonstrate that while ADAMTS-12 is coexpressed in COMP-producing musculoskeletal tissues, it has a wider tissue distribution (3,4,38,46).

Increased Expression of ADAMTS-12 in the Cartilage and Synovium of Patients with Arthritis—To determine whether the expression of ADAMTS-12 in cartilage and synovium is altered in OA or RA, a quantitative real-time PCR was performed using a sequence-specific probe and primers for ADAMTS-12. Total RNA was extracted from adult age-matched normal and arthritic tissues (articular cartilage and synovium). As shown in Fig. 6B, ADAMTS-12 mRNA was significantly upregulated in both OA and RA cartilage ($p < 0.05$ and $p < 0.001$, respectively) compared with the normal control. Further analysis of synovium samples revealed that the level of ADAMTS-12 was also significantly up-regulated in RA synovium compared with normal synovium ($p < 0.001$, Fig. 6C).

DISCUSSION

The present study demonstrates that ADAMTS-12 binds to the EGF-like repeat domain of COMP via its four C-terminal TSP-1 like repeats and that ADAMTS-12-mediated COMP degradation is Zn^{2+}- and pH-dependent (Figs. 3-5). In view of the fact that prominent COMP degradative fragments in OA and RA are produced by cleavage within the COMP EGF-like molecular domain, the binding of ADAMTS-12 to this same region with subsequent COMP cleavage is strong evidence that ADAMTS-12 plays an important role in COMP degradation (13).

COMP interacts with multiple protein partners; these interactions are important for the COMP physiologic functions and cytoplasmic processing and transport. COMP can mediate chondrocyte attachment through interactions with integrins. Through these interactions, COMP may be able to regulate cellular activities and respond to environment in the surrounding cartilage matrix (3,47). Several reports suggest that COMP may function to stabilize the articular cartilage extracellular matrix by specific cation-dependent interactions with matrix components, including collagen types II and IX, fibronectin, aggrecan, and matrilin-1, -3, and -4 (10,48-51). COMP has also been shown to associate with several chaperone proteins, including BiP, calreticulin, protein disulfide, ERp72, Grp94, HSP47, and calnexin, and it has been proposed that these associations facilitate the processing and transport of wild-type COMP in normal chondrocytes and in the retention of mutant COMP in pseudoachondroplasia chondrocytes (52-54). In addition to the interactions between COMP and its protein partners, the five-stranded N-terminal domain of COMP forms a complex with vitamin D-3, illustrating that COMP has storage function for hydrophobic compounds, including prominent cell signaling molecules (55).

Purified COMP has been reported to be digested *in vitro* by several members from the family of matrix metalloproteinases (MMPs), including interstitial collagenase (MMP-1), collagenase-3 (MMP-13), strome-lysin-1 (MMP-3), gelatinase-B (MMP-9), MMP-19, and enamelysin (MMP-20) (56). It was also shown that ADAMTS-4 is able to cleave purified COMP in an *in vitro* digestion assay and that the resultant fragment has a molecular mass similar to one of the fragments observed in OA and RA samples (57); all these assays, however, were performed using an *in vitro* digestion system in which both enzymes and substrates were at higher concentrations than those in physiological/pathological conditions. None of these metalloproteinases has been found capable of associating with COMP, which is probably

necessary for COMP degradation *in vivo*. Results from this study using ADAMTS-12 comprehensive protein-protein interactions and enzymatic activities assays clearly show that ADAMTS-12 can bind to and cleave COMP (Figs. 1-5).

The C-terminal domain of metalloproteinases were found to be important for binding substrates and determining enzyme selectivity; data from chimeric constructs indicate that collagenases, stromelysins, and gelatinases interact with their macromolecular substrates via this domain (see the review by Martel Pelletier, Ref. 62). The matrix-binding properties of ADAMTS-1 appear to be related to the number of TSP repeats in its C-terminal region (63), and our finding that four C-terminal TSP repeats of ADAMTS-12 are required and sufficient for binding to COMP also supports this concept. Recent studies also provide evidence that ADAMTS-4 interacts with aggrecan via its cysteine-rich/spacer domains (64), whereas its C-terminal region plays a major role in regulating aggrecanase activity by masking its general proteolytic activity (65).

In addition to their substrates, the enzymatic activities of ADAMTS proteins may be regulated by their associated proteins, and several ADAMTS binding partners have been isolated. α_2-Macroglobulin was found to form protein complexes with ADAMTS-4 and ADAMTS-5 and represents an endogenous inhibitor of these enzymes (58). It was also reported that the aggrecanase activity of ADAMTS-4 is inhibited by fibronectin through interaction with its C-terminal domains, suggesting that this extracellular regulation mechanism of ADAMTS-4 activity may be important for the degradation of aggrecan in arthritic cartilage (59). ADAMTS-4 also associates with α_1-antitrypsin a member of the family of plasma serine proteinase inhibitors, but the physiological significance of the interaction between them remains unclear (60). The extracellular matrix protein fibulin-1 was identified as an ADAMTS-1 interacting molecule in a yeast-two-hybrid screen, and fibulin-1 was found to enhance the capacity of ADAMTS-1 to cleave aggrecan, indicating that fibulin-1 is a regulator of ADAMTS-1-mediated proteoglycan proteolysis and thus may play an important role in proteoglycan turnover (61).

As shown in Fig. 5, *D* and *E*, the COMP-degrading activity of ADAMTS-12 depends on the presence of appropriate cations; this dependence was further verified with EDTA chelator, since 5 mM EDTA totally abolished COMP digestion mediated by ADAMTS-12 (not shown). ADAMTS-12 demonstrated highest enzymatic activities in the presence of 2 mM Zn^{2+}, whereas most metalloproteinases usually require lower concentrations of Zn^{2+} for cleaving their substrates (*e.g.* 0.1 mM). The differences in metal requirements between metalloproteinases may indicate a difference in the cation dependence of these enzymes. A good example in this regard is the matrix metalloproteinases gelatinases. The 68,000 and 130,000 gelatinases are active at higher concentration of Zn^{2+} (2 mM), but 60,000 gelatinase is active at a very low concentration of Zn^{2+} (5 μM) (45).

Reverse-transcription PCR was employed to examine the expression of ADAMTS-12 in human musculoskeletal tissues and revealed that ADAMTS-12 is expressed in COMP-expressing tissues, including cartilage, synovium, and tendon (Fig. 6A). A real time PCR assay performed to compare the expression profile of the ADAMTS genes in OA and normal cartilage showed that ADAMTS-12 mRNA was significantly up-regulated in OA cartilage ($p < 0.05$) (1). This finding is in agreement with our quantitative real time PCR assays using OA cartilage; we also found, however, that ADAMTS-12 is significantly up-regulated in cartilage and synovium obtained from patients with RA ($p < 0.001$, Fig. 6, *B* and *C*), suggesting that ADAMTS-12 plays an important role in joint degenerative disease progression. The increased expression of ADAMTS-12 in the joint tissues of arthritic patients may be caused by proinflammatory cytokines, including TNF-α and IL-1β (not shown).

Our identification of ADAMTS-12 as a COMP-binding protein and subsequent characterization of the enzyme/substrate association and COMP degradation mediated by ADAMTS-12 significantly extend our understanding of the degradative events that occur in joint disorders and promise to increase our ability to monitor the biological and physical properties of cartilage extracellular matrix. Because the levels of ADAMTS-12 are significantly increased in the cartilage and synovium of arthritis patients, this enzyme appears to play an important role in the pathophysiology of cartilage degradation in arthritis.

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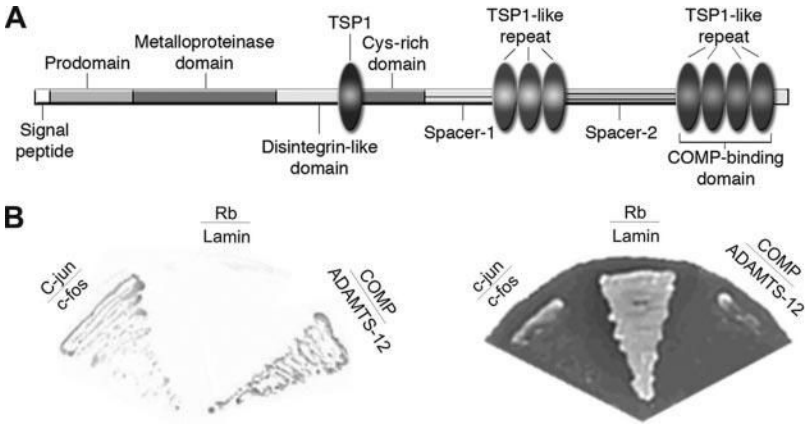

FIGURE 1.

Binding of COMP to ADAMTS-12 in yeast. *A*, domain organization of ADAMTS-12. The C-terminal COMP binding region is indicated. *B*, yeast two-hybrid assay to test the interaction of proteins fused to the VP16 AD and proteins fused to the Gal4 DBD. Each pair of plasmids, as indicated, encoding proteins fused to VP16 (*below the line*) in the vector pPC86 (*i.e.* pPC86-c-Jun, pPC86-ADAMTS-12, and pPC86-Rb) and those encoding proteins fused to Gal4 (*above the line*) in the vector pDBleu (*i.e.* pDB-c-Fos, pDB-COMP, and pDB-lamin) were cotransfected into yeast strain MAV203. Yeast transformants were selected on S.D.-leu$^-$/trp$^-$ plates and tested for β-galactosidase activity (*left panel*) and for growth on plates containing 5-fluoroorotic acid (*5FOA*)(*right panel*, S.D.-leu$^-$/trp$^-$/5FOA$^+$). The known interaction between c-Jun and c-Fos is used as a positive control, and the lack of interaction between Rb and lamin as a negative control.

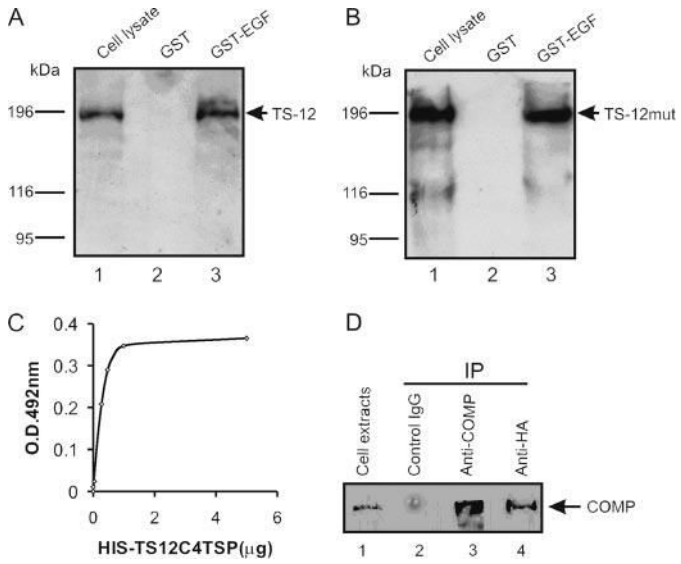

FIGURE 2.

COMP associates with ADAMTS-12 both *in vitro* and *in vivo*. *A* and *B*, GST pull-down assay. Purified GST or GST-EGF fusion protein immobilized on glutathione-Sepharose beads were incubated with cell extracts bearing either HA-tagged wild-type ADAMTS-12 (TS-12, *A*) or ADAMTS-12 with mutant catalytic domain (TS-12mut, *B*). Proteins trapped by the EGF-like domain of COMP fused to GST were examined by immunoblotting with anti-HA probe. Cell lysates were used as positive controls. *Arrows* indicate the wild-type ADAMTS-12 (*A*) and its mutant (*B*) bands. *C*, solid phase assay. Various amounts of recombinant His-tagged C-terminal TSP motifs of ADAMTS-12 (His-TS12C4TSP) were immobilized on solid-phase 96-well microtiter plates. After blocking, COMP was added to each well, followed by the addition of 10 mM $CaCl_2$. Samples were then allowed to bind overnight at 4 °C. Bound protein from the liquid phase was detected using monoclonal antibodies to the bound COMP. *D*, coimmunoprecipitation assay. Cell extracts prepared from the COMP stable line transfected with ADAMTS-12HA plasmid were incubated with control IgG (*lane 2*), anti-COMP (*lane 3*), or anti-HA (*lane 4*) antibodies followed by protein A-agarose. The immunoprecipitated protein complex and cell extracts (*lane 1*, a positive control) were examined by immunoblotting with anti-COMP antiserum.

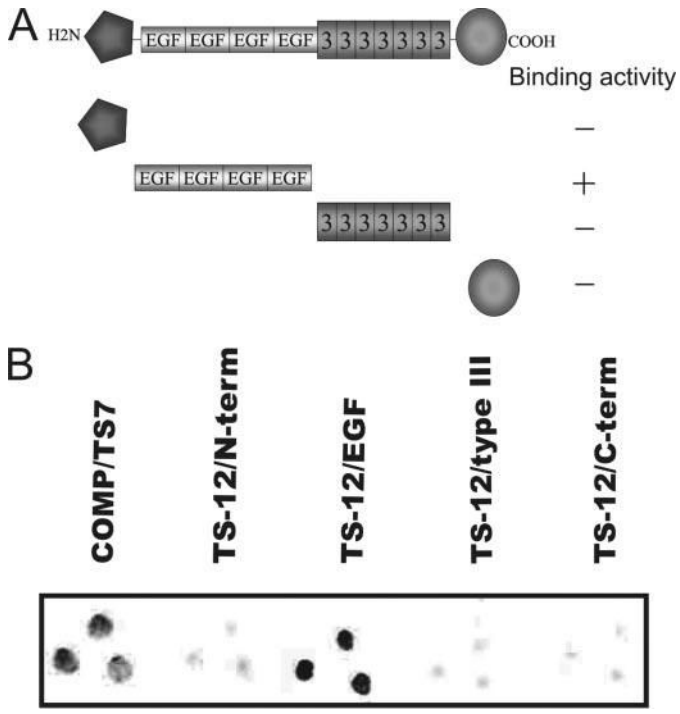

FIGURE 3.

ADAMTS-12 selectively binds to the EGF-like domain of COMP. *A*, schematic structure of COMP constructs used to map those domains (N-terminal, EGF-like, type III, and C-terminal) that bind to ADAMTS-12. Presence or absence of binding between COMP domains and ADAMTS-12 is indicated a "+"or"-" respectively. *B*, β-galactosidase activity was used to test interaction between the C-terminal domain of ADAMTS-12 and one of the four domains of COMP. Three independent yeast transformants for each pair of plasmids were transferred onto a nitrocellulose membrane and the β-galactosidase activity determined. ADAMTS-7 (*TS7*) and COMP, which are known to bind to each other, were used as a positive control.

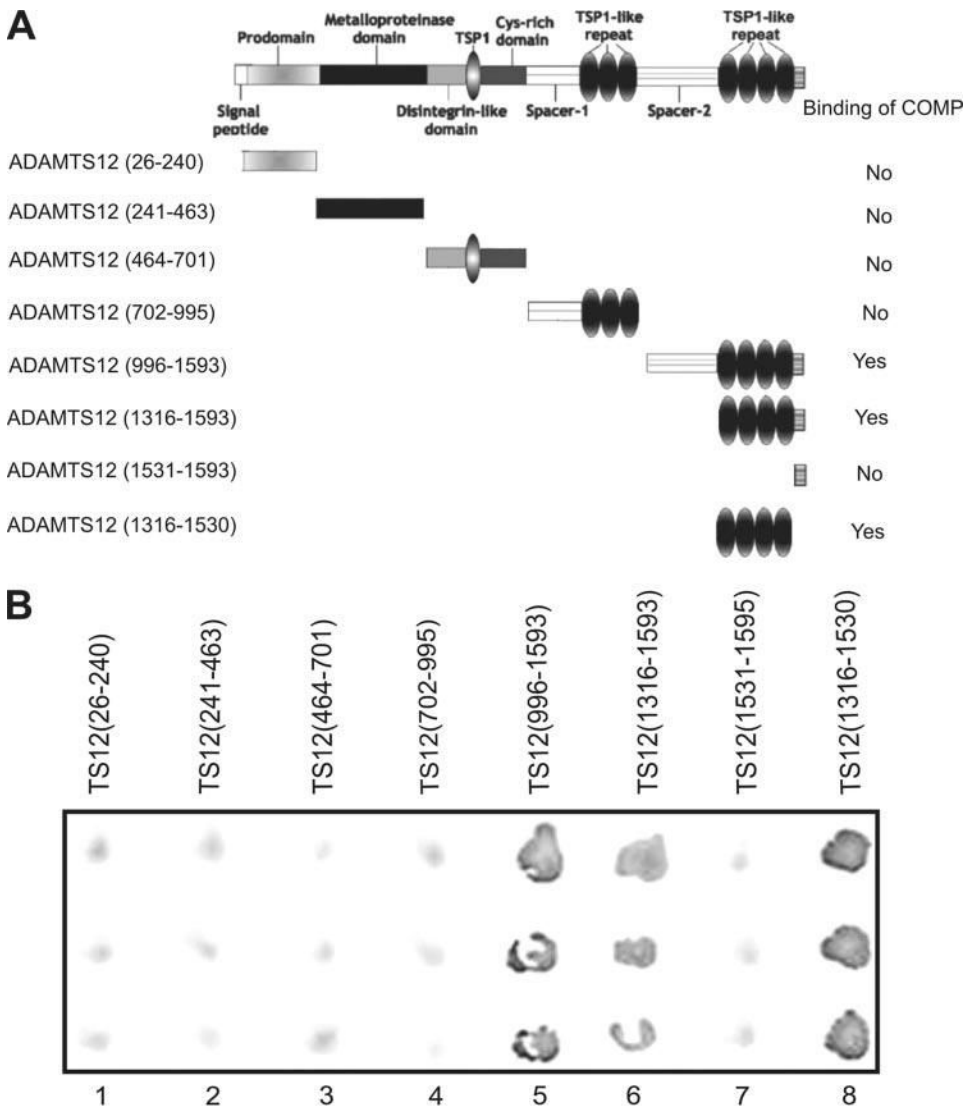

FIGURE 4.

Four C-terminal TSP motifs of ADAMTS-12 are necessary and sufficient for interaction with COMP. *A*, schematic diagram of ADAMTS-12 constructs used to map those of its fragments that bind to COMP. Numbers refer to amino acid residues in ADAMTS-12; *ovals*, TSP motifs. Interactions between COMP and ADAMTS-12 derivatives are summarized and indicated by *Yes* or *No*. *B*, β-galactosidase activity was used to test the interaction between ADAMTS-12 derivatives and COMP. Three independent yeast transformants for each pair of plasmids were transferred onto a nitrocellulose membrane and the β-galactosidase activity determined.

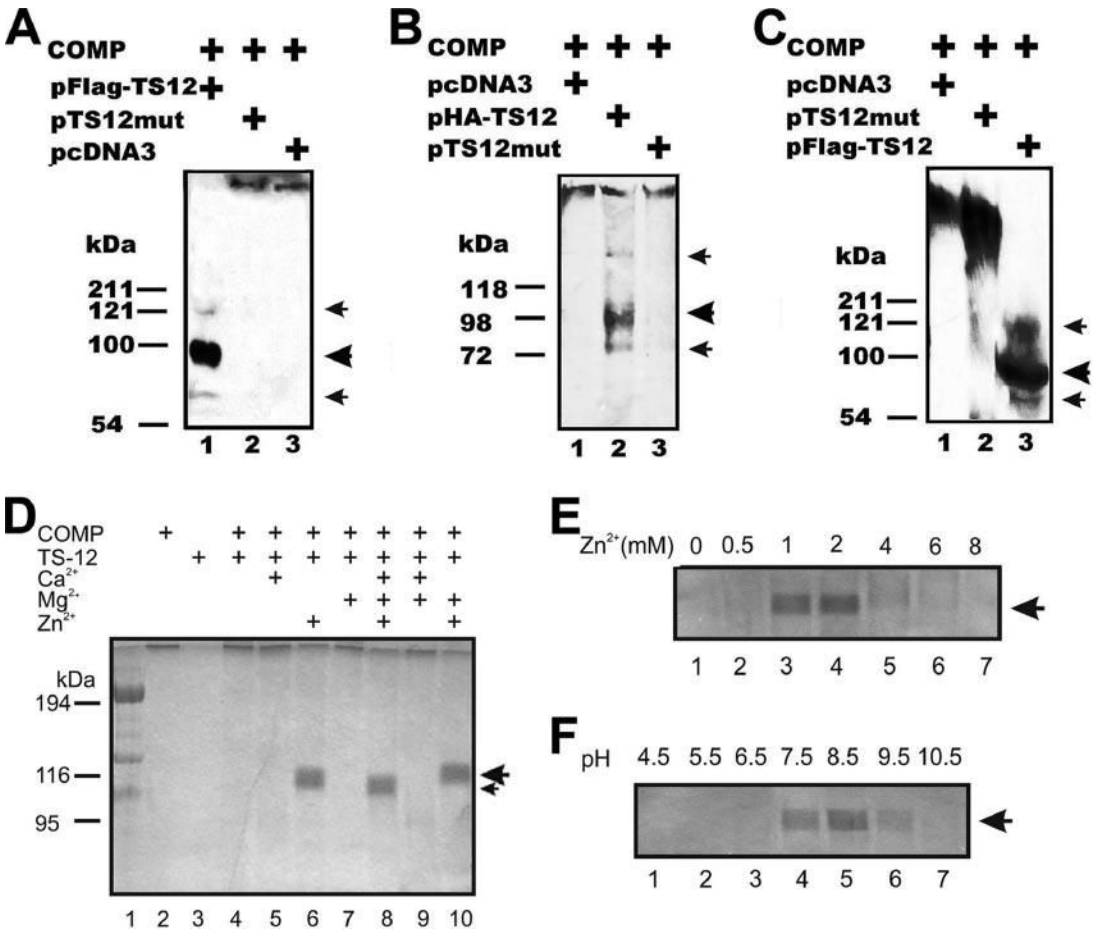

FIGURE 5.

***In vitro* digestion assays of COMP mediated by ADAMTS-12.** *A*, COMP digestion by ADAMTS-12, assayed by cotransfection experiments. The COMP stable line was transfected with either control (*lane 3*) or expression constructs encoding wild-type ADAMTS-12 (pFLAG-TS12, *lane 1*) or ADAMTS-12 with mutant catalytic domain (pTS-12mut, *lane 2*). The media were subjected to nonreduced 8% SDS-PAGE and detected with anti-COMP polyclonal antiserum. The resultant fragments are indicated by *arrowheads*. *B*, COMP digestion by ADAMTS-12, assayed with purified COMP and cell lysates bearing ADAMTS-12. Purified COMP was incubated with cell lysates transfected with control vector (pcDNA3, *lane 1*), a construct encoding intact ADAMTS-12 (pHA-TS12, *lane 2*), or a construct encoding a mutant ADAMTS-12 with a mutant catalytic domain (pTS-12mut, *lane 3*). The cleaved products were subjected to 10% nonreduced SDS-PAGE and analyzed as described in *A*. *C*, COMP digestion by ADAMTS-12, assayed with small scale purified ADAMTS-12. Anti-FLAG M2 antibody was added into the medium harvested from cells transfected with either control (*lane 1*) or expression constructs encoding wild-type ADAMTS-12 (pFlag-TS12, *lane 3*) or ADAMTS-12 mutant (pTS-12mut, *lane 2*), followed by addition of protein A-agarose. Purified ADAMTS-12 or its mutant was incubated with purified COMP, and the digestion was performed as described in *B*. *D*, to demonstrate that Zn^{2+} is essential for the enzymatic activity of ADAMTS-12, purified COMP was incubated with purified catalytic domain of ADAMTS-12 in a digestion buffer supplemented with 5 mM $CaCl_2$,2mM $ZnCl_2$, 2.5 mM $MgCl_2$, or a combination of these, as indicated; the digested proteins were resolved by 10% nonreduced SDS-PAGE and the gel stained with Coomassie Brilliant Blue G colloidal solution. The *large arrow* indicates the resultant fragment; the *small*

arrow indicates the additional (lower) band in *lane 8*, which is probably caused by the conformation change in the presence of Ca^{2+}. *E*, to demonstrate that ADAMTS-12 activity depends on the appropriate concentration of Zn^{2+}, the same digestion was performed in a buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$, pH7.5) containing various concentration of Zn^{2+} (0, 0.5, 1.0, 2.0, 4.0, or 8.0 mM), as indicated; digested proteins were resolved as in *D*. *F*, to demonstrate that the activity of ADAMTS-12 is pH-dependent, the same digestion was performed in a buffer supplemented with 5 mM $CaCl_2$,2mM $ZnCl_2$, and 2.5 mM $MgCl_2$ at various pH values, as indicated; digested proteins were resolved as in *D*.

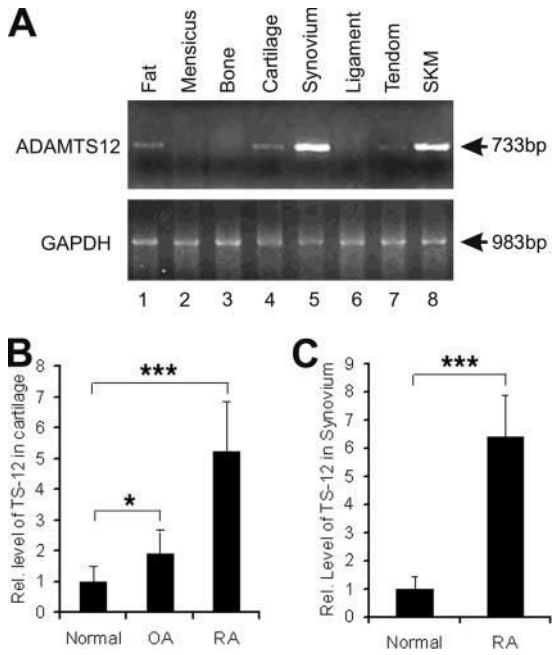

FIGURE 6.

Increased expression of ADAMTS-12 in the cartilage and synovium of arthritis patients. *A*, expression assay of ADAMTS-12 in human musculoskeletal tissues by RT-PCR. Amplification products are consistent with a predicted size of 733 bp for ADAMTS-12 and 983 bp for GAPDH. *SKM*, skeletal muscle; Lanes marked *M* contain the DNA marker. *B*, expression of ADAMTS-12 in normal, OA, and RA cartilage assayed by real time PCR; expression of ADAMTS-12 in each sample was normalized against the 18 S rRNA endogenous control. The normalized values were then calibrated against the normal cartilage value. *C*, expression of ADAMTS-12 in normal and RA synovium. Samples were processed and data analyzed as in *B*. The units are arbitrary, and the *leftmost bar* in each panel indicates a relative level of ADAMTS-12 of 1. *, $p < 0.05$; ***, $p < 0.001$ *versus* normal control.