Published in final edited form as:
FASEB J. 2006 May ; 20(7): 988–990.

ADAMTS-7: a metalloproteinase that directly binds to and degrades cartilage oligomeric matrix protein

Chuan-ju Liu[*,†,1,2], **Wei Kong**[*,1], **Kiril Ilalov**[*], **Shuang Yu**[‡], **Ke Xu**[*], **Lisa Prazak**[*], **Marc Fajardo**[*], **Bantoo Sehgal**[*], and **Paul E. Di Cesare**[*,†,2]

[*]*Musculoskeletal Research Center, New York University-Hospital for Joint Diseases Department of Orthopaedic Surgery, New York, USA;*

†*Department of Cell Biology, New York University School of Medicine, New York, New York, USA;*

‡*Department of Medicine, Yale University School of Medicine, New Haven, Connecticut, USA*

Abstract

Degradative fragments of cartilage oligomeric matrix protein (COMP) have been observed in arthritic patients. The physiological enzyme(s) that degrade COMP, however, remain unknown. We performed a yeast two-hybrid screen (Y2H) to search for proteins that associate with COMP to identify an interaction partner that might degrade it. One screen using the epidermal growth factor (EGF) domain of COMP as bait led to the discovery of ADAMTS-7. Rat ADAMTS-7 is composed of 1595 amino acids, and this protein exhibits higher expression in the musculoskeletal tissues. COMP binds directly to ADAMTS-7 *in vitro* and in native articular cartilage. ADAMTS-7 selectively interacts with the EGF repeat domain but not with the other three functional domains of COMP, whereas the four C-terminal TSP motifs of ADAMTS-7 are required and sufficient for association with COMP. The recombinant catalytic domain and intact ADAMTS-7 are capable of digesting COMP *in vitro*. The enzymatic activity of ADAMTS-7 requires the presence of Zn^{2+} and appropriate pH (7.5-9.5), and the concentration of ADAMTS-7 in cartilage and synovium of patients with rheumatoid arthritis is significantly increased as compared to normal cartilage and synovium. ADAMTS-7 is the first metalloproteinase found to bind directly to and degrade COMP.—Liu, C., Kong, W., Ilalov, K., Yu, S., Xu, K., Prazak, L., Fajardo, M., Sehgal, B., Di Cesare, P. E. ADAMTS-7: a metalloproteinase that directly binds to and degrades cartilage oligomeric matrix protein. *FASEB J.* 20, E129 -E140 (2006)

Keywords

degradation; arthritis; COMP

The extracellular matrix (ECM) of cartilage consists of several types of collagens, proteoglycans, and other noncollagenous macromolecules, all of which interact to form a highly specialized connective tissue (1). Early extracellular cartilage matrix degeneration in arthritis is the result of the action of degradative enzymes. As the severity of arthritis progresses, the synthesis and secretion of matrix-degrading enzymes markedly increase (2). The control

[1]These authors contributed equally to this work.

[2]Correspondence: Department of Orthopaedic Surgery, New York University School of Medicine, 301 East 17th St., New York, NY 10003, USA. E-mail: Chuanju.liu@med.nyu.edu; or Muscu- loskeletal Research Center, Hospital for Joint Diseases, 301 East 17th St., New York, NY 10003, USA. E-mail: pedicesare@aol.com doi: 10.1096/fj.05-3877fje

The authors thank the Musculoskeletal Transplant Foundation for providing human tissue. This work was supported by National Institutes of Health research grant AR052022- 01A1 (C.J.L.) and New York Chapter of the Arthritis Foundation Dorothy W. Goldstein Young Scholar Award (C.J.L.).

of these enzymes is complex, with regulation occurring at three different levels: synthesis and secretion, activation of latent enzyme, and inactivation by proteinase inhibitors.

Cartilage oligomeric matrix protein (COMP), a prominent noncollagenous component of cartilage, accounting for ~1% of the wet weight of tissue, has also been localized in tendon, bone (osteoblasts only), and synovium (3-6). COMP is a 524 kDa pentameric, disulfide-bonded, multidomain glycoprotein composed of approximately equal subunits (~110 kDa each; refs 3,7). Monitoring of COMP levels in either joint fluid or serum can be used to assess the presence and progression of arthritis (8-13). Mutations in the human COMP gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia, autosomal-dominant forms of short-limb dwarfism characterized by short stature, N facies, epiphyseal abnormalities, and early-onset osteoarthritis [(OA); refs 14-20].

Fragments of COMP have been detected in diseased cartilage, synovial fluid, and serum of patients with knee injuries, posttraumatic, primary OA, and rheumatoid arthritis (RA; refs 21-23). The nature of COMP degradation and the enzyme(s) responsible for the production of degradative fragments *in vivo*, however, have yet to be identified. Theoretically, inhibition of degradative enzymes can slow down or block disease progression. The isolation of cartilage degradative enzymes is therefore of great interest from both a pathophysiological and a therapeutic standpoint.

To isolate the physiological enzymes that degrade COMP *in vivo*, we performed a genetic screen based on the yeast two-hybrid screen (Y2H) system that has proven to be an effective tool for identifying protein-binding partners (24-26). Four functional domains of COMP (N-terminal, EGF repeat, type III, and C-terminal domain) were used as bait to screen a rat brain cDNA library. These experiments identified ADAMTS-7, which belongs to the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family (27,28), as a COMP binding partner. The ADAMTS family consists of secreted zinc metalloproteinases with a precisely ordered modular organization that includes at least one thrombospondin type I repeat (29). Important functions have been established for several members of the 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 (28,30-33). ADAMTS-1 and ADAMTS-4 also participate in the turnover of the proteoglycans versican and brevican in blood vessels (34) and the nervous system, respectively (35). ADAMTS-1 has angioinhibitory activities (36) and is essential for the development and function of the urogenital system (37). ADAMTS-2, ADAMTS-3, and ADAMTS-14 are procollagen N-propeptidases (38,39). ADAMTS-2 mutations cause dermatosparaxis, an inherited disorder characterized by severe skin fragility (40). ADAMTS-13 is a von Willebrand factor-cleaving protease, and its mutations lead to inherited life-threatening thrombocytopenic purpura (41).

In this study, we report the identification of ADAMTS-7 as a 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-7 mRNA in RA cartilage and synovium.

MATERIALS AND METHODS

Plasmid constructs

Yeast expression vectors pDBleu and pPC86 (both Life Technologies, Gaithersburg, MD) are fusion vectors for the linkage of proteins to the Gal4 DNA binding domain and to the VP16 transactivation domain, respectively. The fragments encoding the four functional domains, i.e., the N-terminal (aa 20 - 83), EGF repeat domain (aa 84 -261), type III repeat domain (aa 266 -520), and C-terminal (aa 521-755; GenBank accession number AF257516), of mouse COMP

were amplified by polymerase chain reaction (PCR) and cloned inframe into the *Sal*I/*Not*I sites of pDBleu (pDB-COMP-NT, pDB-COMP-epidermal growth factor, pDB-COMP-type III, and pDB-COMP-CT) to serve as bait in the screening assay.

cDNA inserts encoding the fragments (Table 1) of rat ADAMTS-7 were cloned inframe into the *Sal*I/*Not*I sites of pPC86 vector to generate the indicated plasmids.

The bacterial expression vector pGEX-3X (Life Technologies) was used to produce recombinant glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*. The cDNA fragments encoding a catalytic domain-containing segment of hADAMTS-7 (aa 217-468, Genbank accession number AF140675), a C-terminal region (aa 1140 -1595), and a segment from spacer-2 (aa 1174 -1275) of rADAMTS-7 were inserted inframe into the *Bam*HI/*Eco*RI sites of pGEX-3X to generate the plasmids pGEX7-cluster of differentiation, pGEX7-CT, and pGEX7 (aa 1174 -1275), respectively. The bacterial expression pBAD TOPO vector (Invitrogen, Carlsbad, CA) was used to produce His-tagged proteins in *E. coli*.A cDNA segment encoding the four C-terminal TSP motifs (His-TS7C4TSP) of rADAMTS-7 (aa 1140 -1537) 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 basic local alignment search tool (BLAST) software (available at http://www.ncbi.nlm.nih.gov/basic local alignment search tool/).

Expression and purification of GST and His-tagged proteins

For expression of GST fusion proteins, the appropriate plasmids pGEX7-CD, pGEX7-CT, and pGEX7 (aa 1174 - 1275) were transformed into *E. coli* DH5α (Life Technologies). Fusion proteins were affinity-purified on GSH-agarose beads, as described previously (42). To cleave off and remove the GST moiety from the GST fused catalytic domain of ADAMTS-7, 50 μg of purified GST-TS7-CD fusion protein were incubated with 1 μg of Xa factor (New England Biolabs, Beverly, MA) in 20 μl of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 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 GSH-Sepharose 4B beads (Amersham, Piscatway).

His-TS7C4TSP was purified by affinity chromatography using a HiTrap chelating column (Amersham Pharmacia Biotech, Uppsala, Sweden). 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, and 0.05% Brij 35) containing 10 mM imidazole, and the His-TS7C4TSP was eluted with HSB buffer containing 300 mM imidazole.

Cloning and expression of human ADAMTS-7 in eukaryotic cells

The Bac-to-Bac Baculovirus Expression System (Invitrogen) was used to express intact human ADAMTS-7 in insect cells per the manufacturer's protocol. Briefly, the cDNA fragment encoding the full-length human ADAMTS-7 was amplified by PCR and inserted into the *Eco*RI/*Not*I sites of the pFastBac vector, and the resultant plasmid pFastBac-TS-7 was verified by DNA sequencing. Plasmid pFastBac-TS-7 was then transformed into DH10BacTM *E. coli* for transposition into bacmid. Recombinant bacmid DNA was isolated and verified by ADAMTS-7-specific primers. For generating the baculovirus expressing human ADAMTS-7, recombinant bacmid DNA was transfected into Sf9 insect cells and P1 virus stock was isolated and amplified. The presence of human ADAMTS-7 protein in the medium and cell lysates infected with ADAMTS-7 bacluovirus was confirmed by an immunoblotting assay.

Preparation and purification of an antiserum to ADAMTS-7

The GST-ADAMTS-7 (1174 -1275) fusion protein was expressed in *E. coli* DH5α, purified on a GSH-Sepharose column, and subjected to preparative-scale SDS-PAGE. The major band was excised and used to immunize rabbits for polyclonal antiserum production [Zymed Custom Antibody (Ab), Zymed Laboratories, South San Francisco, CA]. To affinity-purify anti-ADAMTS-7 antibodies, the anti-GST activity in the rabbit antiserum was depleted using GST protein immobilized on GSH-agarose beads. The depleted serum was incubated with Affi-Gel-10 beads (Bio-Rad, Hercules, CA) to which purified GST-ADAMTS-7 (aa 1174 -1275) was covalently linked. The bound antibodies were eluted from the beads with 0.15 M glycine buffer (pH 2.5) and immediately neutralized with 1.5 M Tris-HCl buffer (pH 8.0; ref 43).

Y2H library screen

Plasmid pDB-COMP-epidermal growth factor (see above) was used as bait to screen Y2H rat brain cDNA library (Life Technologies) according to a modified manufacturer's protocol. Briefly, bait plasmid was introduced into a yeast MAV203 strain that contained three reporter genes, HIS^+, URA^+, and Lac Z (Life Technologies), and transformants were selected on defined medium lacking leucine. The rat brain cDNA library in the vector pPC86 was then transformed into the resultant Leu^+ yeast strain and plated on medium lacking tryptophan, leucine, histidine, and uracil but containing 25 mM 3-amino-1,2,4-trizone that can specifically inhibit the activity of HIS3 gene product and block the basal concentration of HIS3 in yeast ($sd\text{-}leu^-/trp^-/his^-/ura^-/\ 3AT^+$). After incubation for 7-10 days at 30°C, colonies were screened for β-galactosidase by a filter lift assay (24). Individual pPC86 recombinant plasmids, which were identified in the initial screen were further verified for interaction with bait by repeating the Y2H assay.

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 (44). Total RNA was extracted by the acid-guanidium thiocyanate-phenol-chloroform single-step method followed by RNAeasy kit (Qiagen, Valencia, CA). Total RNA was also extracted from the rat chondrocyte sarcoma (RCS) cell line and from 10 different rat tissues (Ambion, Austin, TX).

One microgram of total RNA per sample was reverse-transcribed using the ImProm-II Reverse Transcription system (Promega, Madison, WI). The following isoform-specific primers were synthesized: 5′- GAGCCTGTCTGGATCCAGCT- GCTGTTC-3′ and 5′- TGCACACCTCTGCCGCAGGTGACT- GTG-3′ for human ADAMTS-7;

5′- GCAACGCTATTGATGAGGAAGACC-3′ and 5′- TTGG-GAAGGGCAGGTGATGTAGGA-3′ for rat ADAMTS-7. The following pairs of oligonucleotides were used as internal controls: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′, for human GAPDH; 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ for rat GAPDH. PCR was performed for 35 cycles (94°C 1 min, 60°C 1 min, 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).

Assay of protein-protein interactions using the Y2H 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. (26) and Hollenberg et al. (24) were followed for *1*) growing and transforming the yeast strain MAV203 with the selected plasmids; and *2*) testing β-galactosidase activity and growth phenotypes on (sd-leu$^-$/trp$^-$/his$^-$/ura$^-$/3AT$^+$) plates and on plates containing 5-fluoroorotic acid (sd-leu$^-$/trp$^-$/5FOA$^+$).

In vitro GST pulldown assay

For examination of the binding of COMP to the C terminal of rADAMTS-7 *in vitro*, GSH-Sepharose beads (50 μl) preincubated with either purified GST (0.5 μg, serving as control) or GST-ADAMTS-7-CT (0.5 μg) were incubated with 0.5 μg of human COMP (purified from HEK293 cells stably transfected with an expression plasmid encoding full-length human COMP) in 150 μl of buffer acetoxymethyl ester (10 mM Tris-HCl, pH. 7.9, 10% glycerol, 100 mM KCl, and 0.5 mg/ml BSA). To examine whether divalent cations were involved in this association, 5 mM Ca^{2+} were added to one set of binding buffer. The bound proteins were denatured in sample buffer and separated by 12% SDS-PAGE, and COMP protein was detected by Western blotting with polyclonal rabbit anti-COMP antiserum (4,21,45).

Solid-phase binding assay

Microtiter plates (96-well; enzyme immunoassay/RIA plates, Costar, Badhoevedorp, The Netherlands) were coated with various amounts (0.001-5.000 μg) of purified His-TS7C4TSP in 100 μl of TBS buffer (50 mM Tris-HCl, and 150 mM NaCl, pH 7.4) overnight at 4°C. Wells were blocked with 1% BSA in TBS buffer for 3 h at 37°C. After being washed with TBS and 0.05% Tween, 100 μl of 50 μg/ml of COMP were 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 anti-mouse Ab conjugated with horseradish peroxidase (Antigenix America, Huntington Station, NY) and 5-amino-2-hydroxybenzoicacid as a substrate, with absorbance measured at 492 nM in an ELISA reader.

Coimmunoprecipitation

Slices of articular cartilage (1-2 g wet wt) from the knee of a 58-yr-old deceased patient without knee arthritis (obtained from the Musculoskeletal Transplant Foundation and immediately frozen en bloc) were ground in liquid nitrogen using a freezer mill (Spex Industries, Edison, NJ) to yield a fine particulate. Cartilage was extracted with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, containing the proteinase inhibitors 1 mM phenylmethanesulfonyl fluoride, 2 mM *N*-ethylmaleimide, and 0.025 mg/ml leupeptin). Approximately 500 μg of cartilage extract were incubated with anti-COMP (25 μg/ml) or control rabbit IgG (25 μg/ml) antibodies for 1 h, followed by incubation with 30 μl of protein A-agarose (Life Technologies) at 4°C overnight. After being washed five times with immunoprecipitation buffer, bound proteins were released by boiling in 20 μl of 2× SDS loading buffer for 3 min (46). Released proteins were examined by Western blotting with anti-ADAMTS-7 antibodies, and the signal was detected using the enhanced chemiluminescence chemiluminescent system (Amersham Pharmacia Biotech).

In vitro digestion assay

To determine whether the catalytic domain of ADAMTS-7 can digest COMP *in vitro*, purified COMP (200 nM) was incubated with either various amounts of purified bacteria-expressed catalytic domain of ADAMTS-7 in a digestion buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 2 mM $ZnCl_2$, and 0.05% Brij-35, pH 7.5) at 37°C for 12 h. The digested products were

resolved by 10% nonreduced SDS-PAGE and intact COMP, and fragments were visualized with Coomassie brilliant blue G-colloidal solution (Sigma, St. Louis, MO) according to the manufacturer's recommendations.

To examine whether the enzymatic activity of ADAMTS-7 depends on the divalent cations, purified COMP substrate (250 nM) was incubated with purified catalytic domain of ADAMTS-7 (25 nM) in digestion buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) supplemented with 5 mM $CaCl_2$, 2 mM $ZnCl_2$, 2.5 mM $MgCl_2$, 5 mM EDTA, or various combinations at 37°C for 12 h. The digested products were resolved by 10% nonreduced SDS-PAGE, and the gel was stained with Coomassie brilliant blue G-colloidal solution.

To test the enzymatic activity of ADAMTS-7 at different pHs, the same digestion was performed in a buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, 2 mM $ZnCl_2$, and 2.5 mM $MgCl_2$) at various pH values (4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, and 11.5) at 37°C for 12 h and digested proteins were processed as above.

To examine COMP degradation by full-length ADAMTS-7 and to investigate the zinc ion concentration dependence of the intact enzyme, purified COMP (200 nM) was incubated with the cell lysates prepared from Sf9 insect cells infected with either control or ADAMTS-7 bacluovirus in the presence of lower (0.1 mM) or higher (2 mM) levels of $ZnCl_2$ as well as in the absence of Zn^{2+} by addition of 5 mM EDTA in a digestion buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, and 0.05% Brij-35, pH 7.5) at 37°C for 12 h. The digested nonreduced products were resolved by 10% SDS-PAGE, and intact COMP and COMP fragments were detected by Western blotting with polyclonal rabbit anti-COMP antiserum, as described previously (4,21,45).

Expression of ADAMTS-7 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 yr, range 43-64 yr) who had died of diseases unrelated to arthritis (specimens obtained en bloc from the Musculoskeletal Transplant Foundation). The grade of OA was determined using the Kellgren-Lawrence Grading System (47). Normal tissues samples were without radiographic or intra-articular evidence of arthritic disease (Kellgren-Lawrence Grade 0). Arthritic cartilage and synovium were obtained from 12 patients under going 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 yr, range 49 - 66 yr) 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 (48).

After total RNA extraction and reverse transcription, real-time PCR was performed using a sequence-specific probe and primers for ADAMTS-7 {fluorescence-labeled oligonucleotide probes [using 6-carboxy-fluorescein (FAM)] probe: AAG- CGCTTCCGCCTCTGCAACC; primers: CAGCCTACGCCC- AAATACAAA and CCCTTGTAGAGCATAGCGTCAAA}. 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 were obtained. As an internal control, 18S 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 18S 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 vol. 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

Isolation of enzyme(s) responsible for COMP degradation using the Y2H system

To identify the physiological enzymes required for COMP degradation, we performed a genetic screen based on the Y2H system (24-26) and discovered an enzyme that potentially uses COMP as its substrate. Briefly, we linked the four functional domains of COMP, the N-terminal pentamerizing domain (aa 20 - 83), the EGF-like domain (aa 84 -261), type 3 repeats (aa 266 -520), and the C-terminal domain (aa 521-755), to the Gal4 DNA-binding domain (GAL4DBD) in the plasmid pDBleu. We used the respective constructs as bait to screen a library of rat brain cDNA expressed as fusion proteins to the VP16 acidic activation domain (VP16AD) in the vector pPC86. No proteins were isolated when N-terminal and type 3 domains were employed as bait; EPS15 was identified as a COMP-associated protein when the C-terminal domain was used as a bait.

A Y2H rat brain cDNA library was screened with the construct encoding the EGF-like repeats of COMP. We screened ~2.5 million clones and identified 21 that activated the three reporter genes. Further tests involved the retransformation of yeast with the purified target plasmids and bait. Only 12 of the original 21 yeast clones expressed hybrid proteins that still interacted with the EGF-like domain bait (not shown). Four positive clones among them contained 1613 bp identical inserts.

A BLAST search performed using a 1613 bp insert sequence revealed that the insert sequence encoding four TSP type 1 repeats matched a computer-generated complete cDNA sequence of a member of the ADAMTS family (GenBank accession number XM_236471) with a 177 bp internal deletion that represents an intron between exons 20 and 21. An extensive search for rat DNA genomic sequences encoding an uncharacterized 1613 insert and XM_236471 made it possible to identify a rat genomic clone (CH230 -120K20, accession number AC103554). The rat ADAMTS-7 gene is located in rat chromosome 8; its corresponding cDNA sequence is derived from 26 exons (not shown). We obtained the complete cDNA clone of rat ADAMTS-7 via RT-PCR cloning. Our cDNA clone sequence, comprising 5259 bp, was submitted to GenBank and assigned accession number AY327121. Computer analysis of our sequence revealed an open reading frame coding for a protein of 1595 amino acid residues with a predicted molecular mass of 175,814 Dalton (not shown) and a 3′ untranslated region (218 bp) followed by a poly(A) tail.

Musculoskeletal tissues distribution of ADAMTS-7

RT-PCR of ADAMTS-7 detected ADAMTS-7 in rat liver, embryo, ovary, kidney, testicle, lung, and thymus tissues, and its mRNA is qualitatively expressed at lower levels in other tissues such as spleen, heart, and brain (not shown). An RT-PCR assay was also performed to examine the expression of ADAMTS-7 mRNA in eight specimens of normal human musculoskeletal tissue. As seen in Fig. 1, 167-bp hADAMTS-7 fragment was amplified using hADAMTS-7-specific primers from bone, cartilage, synovium, tendon, and ligament, in which COMP was also present (3,49). ADAMTS-7 is also detectable, although at lower levels, in meniscus, skeletal muscle, and fat. These results demonstrate that although ADAMTS-7 is coexpressed in COMP-producing musculoskeletal tissues, it has a wider tissue distribution (3-6).

Confirmation of interaction between COMP and ADAMTS-7 in yeast

The Y2H assay was repeated to verify the interaction between the EGF-like domain of mouse COMP (aa 84 -261) and the C terminal of rat ADAMTS-7 (aa 1139 -1595). For this purpose, the plasmid encoding the EGF-like domain of COMP linked to Gal4DBD (above the line in Fig. 2) and the plasmid encoding the C terminal of ADAMTS-7 fused to the VP16AD (below the line in Fig. 2) 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. The interaction between the C-terminal polypeptide of ADAMTS-7 and the EGF-like domain of COMP was verified by β-galactosidase assay and growth phenotype on selective media (Fig. 2). 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-7 in yeast, based on β-galactosidase activity (Fig. 2, *left*), growth on plates lacking histidine and uracil and containing 3AT (Fig. 2, *center*), 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. 2, *right*.)

Direct binding of COMP to the C-terminal polypeptide of rADAMTS-7 in vitro

The interaction between COMP and ADAMTS-7 was also confirmed using an *in vitro* GST pulldown assay. Briefly, affinity-purified GST and a purified rADAMTS-7 C-terminal (aa 1139-1595) as a GST fusion protein (GST7-CT) that were immobilized on GSH-Sepharose beads were incubated with purified hCOMP (from native cartilage) and after being washed were resolved by Western blotting. Purified GST did not pull down hCOMP protein (Fig. 3A, lane 2), whereas GST7-CT efficiently pulled down the hCOMP (Fig. 3A, lanes 3 and 4), indicating that hCOMP directly binds to the C-terminal of ADAMTS-7; in addition, this association is significantly enhanced by the addition of divalent cations (5 mMCa^{2+}). The interaction of COMP and ADAMTS-7 was also characterized by titration experiments in which COMP showed dose-dependent binding and saturation to the dilution series of solid-phase His-TS7C4TSP (Fig. 3B).

Binding of COMP to ADAMTS-7 in vivo

The *in vivo* interaction between COMP and ADAMTS-7 was verified using a coimmunoprecipitation (CO-IP) assay to determine whether these two proteins are bound in native articular cartilage. Affinity-purified polyclonal antibodies against ADAMTS-7 generated from recombinant GST-ADAMTS-7 (aa 1174 -1275) that was used as an antigen were confirmed to be specific from Western blots performed using recombinant ADAMTS-7 protein and osteoblastic MG-63 cell line extracts (Fig. 3C); a band with an apparent molecular mass of ~200 kDa was resolved. The anti-ADAMTS-7 antibodies did not cross-recognize COMP (Fig. 3C, lane 3). The antigen was a segment from space-2 that was expressed in bacteria in which posttranslation modification is absent; thus this Ab may preferentially recognize the unglycosylated or poorly-glycosylated forms of ADAMTS-7. This may explain the discrepancy in protein mobility and pattern in the gel (50). The polyclonal antiserum against COMP was generated using intact purified hCOMP as an antigen (4,21,45). For the CO-IP assays, the cartilage extractswere incubated with either anti-COMP antiserum (Fig. 3D, lane 2) or control IgG (Fig. 3D, lane 3), and the immunoprecipitated complexes were subjected to a reducing SDS-PAGE and detected with anti-ADAMTS-7 antibodies. A specific ADAMTS-7 band was present in the immunoprecipitated complexes brought down by anti-COMP (lane 2) but not control IgG (lane 3) antibodies, demonstrating that ADAMTS-7 specifically binds to the COMP *in vivo*.

Selective association of ADAMTS-7 with the EGF-like domain of COMP

After ADAMTS-7 was identified as a COMP-binding protein using the Y2H screen, we sought to establish whether in addition to EGF domain, other domains of COMP associate with ADAMTS-7. A filter-based β-galactosidase assay was used to determine whether coexpression of the various domains of COMP/Gal4DBD and ADAMTS-7/VP16AD fusion proteins activate the reporter LacZ gene. As shown in Fig. 4, ADAMTS-7 selectively interacts with only the EGF-like domain of COMP.

Four C-terminal TSP motifs of ADAMTS-7 are required and sufficient for association of COMP

To identify the COMP-binding motif in ADAMTS-7, we generated various constructs that expressed various ADAMTS-7 deletion mutants in yeast. Results from filter-based β-galactosidase assays (Fig. 5B) of all these mutants are summarized in Fig. 5A. Neither the ADAMTS-7 prodomain, nor the metalloproteinase plus disintegrin-like and cysteine-rich domain, nor the spac- er-1 plus three TSP repeats bound to COMP. As expected, the spacer-2 plus four C-terminal TSP 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 two TSP repeats eliminated binding, indicating that TSP repeats in the C-terminal are required. Smaller fragments of this domain were then tested. When only two TSP repeat domains were used, binding was lost. When four TSP repeats were used, however, COMP binding occurred. Our conclusion from this set of experiments is that that four C-terminal TSP repeats of ADAMTS-7 are required and sufficient for its interaction with COMP (Table 1).

Cleavage of COMP by ADAMTS-7 in vitro

It was recently reported (51) that the catalytic domain of ADAMTS-20 produced in bacteria can digest its substrates *in vitro*. Using a similar method, we purified the catalytic domain (aa 217-468) of hADAMTS-7 as a GST fusion protein (GST-TS7-cluster of differentiation) in bacteria. The GST moiety was further removed by an Xa factor, and the purity of proteins was confirmed by visualization using silver staining (Fig. 6A). Recombinant catalytic domain of ADAMTS-7 was incubated with purified human COMP in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$,2mM $ZnCl_2$, and 0.05% Brij-35, pH 7.5. As shown in Fig. 6B and *C*, the catalytic domain of ADAMTS-7 digested COMP in a dose- and time-dependent manner.

The COMP-degrading activity of ADAMTS-7 was also demonstrated by using the recombinant intact enzyme (Fig. 6D): control cell lysates did not show any enzymatic activity (lane 1), whereas cell lysates prepared from ADAMTS-7 bacluovirus-infected insect cells did cleave COMP in the digestion buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% Brij-35, pH 7.5) supplemented with both lower (0.1 mM) and higher (2.0 mM) concentrations of Zn, although ADAMTS-7 appeared to have a slightly higher enzymatic activity in the presence of 2.0 mM $ZnCl_2$ (lanes 2 and 3). In addition, the cleavage of COMP by ADAMTS-7 was blocked in the presence of 5mM EDTA chelator (lane 4). Two additional fragments between 95 and 51 kDa were seen in addition to the 100 kDa fragment that was predominantly produced with the catalytic domain alone, suggesting that intact enzyme may cleave COMP at more than one site. The COMP products degraded by ADAMTS-7 appear to have a molecular mass similar to some of the more prominent fragments that have been resolved from the synovial fluid of patients with arthritic diseases (21).

Cleavage of COMP by ADAMTS-7 is Zn^{2+}- and pH-dependent

Cations are crucial for the enzymatic activity of metalloproteinases. To further determine the cation-dependence of ADAMTS-7-mediated COMP cleavage, purified COMP substrate and

recombinant catalytic domain of ADAMTS-7 were incubated in digestion buffer in the presence or absence of various cations, including Ca^{2+}, Zn^{2+}, and/or Mg^{2+} (Fig. 7A). A degraded COMP fragment (large arrow in Fig. 7A) was detectable in the digestion buffer with Zn^{2+} (lane 3) but was undetectable in the digestion buffer with Ca^{2+} (lane 2) or Mg^{2+} (lane 4) used alone. In the presence of Zn^{2+} (compare lanes 7 and 5), 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-7, since it did not affect the electrophoretic mobility of the COMP-digested fragment (compare lanes 7 and 3). In line with the assay done with the intact ADAMTS-7 enzyme (Fig. 6D, lane 4), EDTA chelator also totally abolished the cleavage of COMP mediated by the recombinant catalytic domain of ADAMTS-7 (Fig. 7A, lane 8).

In vitro digestions were performed at various pH values to examine its regulation of ADAMTS-7 activity. As shown in Fig. 7B, ADAMTS-7 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-7 is pH dependent.

Increased expression of ADAMTS-7 in the cartilage and synovium of patients with RA

To determine whether the expression of ADAMTS-7 in cartilage and synovium is altered in OA and/or RA, a quantitative real-time PCR was performed using a sequence-specific probe and primers for ADAMTS-7. Total RNA was extracted from adult age-matched normal and arthritic tissues (articular cartilage and synovium). As shown in Fig. 8A, ADAMTS-7 mRNA was significantly up-regulated in RA cartilage, and only slightly up-regulated in OA cartilage, compared to the normal control. Further analysis of synovium samples revealed that the concentration of ADAMTS-7 was also significantly up-regulated in RA synovium compared to normal synovium (Fig. 8B).

DISCUSSION

The Y2H screen was used to identify protein interaction partners of COMP, an ECM protein that is prominent in cartilage and undergoes degradation in arthritic diseases. The present study demonstrated that ADAMTS-7 binds to the EGF repeat domain of COMP via its four C-terminal TSP motifs and that recombinant ADAMTS-7 can digest COMP in a dose-, time-, cation-, and pH-dependent manner. In view of the fact that prominent COMP degradative fragments in OA and RA are produced by cleavage within EGF-like molecular domain of COMP, the binding of ADAMTS-7 to this same region with subsequent COMP cleavage is strong evidence that ADAMTS-7 plays an important role in COMP degradation (21).

COMP interacts with multiple protein partners; these interactions are important for the physiological functions and cytoplasmic processing and transport of COMP. COMP appears to mediate chondrocyte attachment via an integrin receptor (5), and several reports suggest that COMP may function to stabilize the articular cartilage ECM by specific cation-dependent interactions with matrix components, including collagen types II and IX, fibronectin, aggrecan, and matrilin-1, -3, and -4 (11,52-55). 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 inthe retention of mutant COMP in pseudoachondroplasia chondrocytes (56-58). In addition to the interactions between COMP and its protein partners, the five-stranded N-terminal domain of COMP forms a complex with vitamin DL-3, illustrating that COMP has storage function for hydrophobic compounds, including prominent cell-signaling molecules (59).

Purified COMP has been reported to be digested by several matrix metalloproteinases (MMPs), including interstitial collagenase (MMP-1), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), gelatinase-B (MMP-9), MMP-19, and enamelysin (MMP-20) (60). Recently, it was found that ADAMTS-4 can also cleave purified COMP *in vitro* (61); in this study, we present evidence showing that both the recombinant catalytic domain and intact ADAMTS-7 also digest COMP *in vitro.* Domain structure alignments between ADAMTS-7 and other members of the ADAMTS family reveal that ADAMTS-7 and ADAMTS-12 share a similar domain organization and that each has four TSP repeats located in the C-terminal, suggesting that ADAMTS-12 may also associate with COMP and may also be a potential COMP-degrading enzyme.

RT-PCR with isoform-specific primers for human ADAMTS-7 showed that ADAMTS-7 exhibits higher expression in musculoskeletal tissues (Fig. 1). In a real-time PCR assay performed by Dr. Ian Clark's laboratory to examine the expression of the ADAMTS-7 gene in OA and normal cartilage, samples began to amplify around Ct39 at low levels, and no significant differences were observed between normal and OA cartilage (personal communication). This finding is in agreement with our quantitative real-time PCR assays on OA cartilage; we also found, however, that ADAMTS-7 is significantly up-regulated in cartilage and synovium obtained from patients with RA (Fig. 8), suggesting that ADAMTS-7 plays an important role in joint degenerative disease progression. The increased expression of ADAMTS-7 in the joint tissues of RA patients is probably due to proinflammatory cytokines, including TNF-α (62).

The C-terminal domains 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 et al. (63)]. The matrix-binding properties of ADAMTS-1 appear to be related to the number of TSP repeats in its C-terminal region (36), and our finding that four C-terminal TSP repeats are required and sufficient for binding to COMP also supports this concept (Fig. 5). 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).

Our identification of ADAMTS-7 as a COMP-binding protein and subsequent characterization of the enzyme/substrate association and COMP degradation mediated by ADAMTS-7 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 ECM. Since ADAMTS-7 concentration is significantly increased in the cartilage and synovium of RA patients, it remains to be determined whether ADAMTS-7 can be employed as a novel biomarker for RA.

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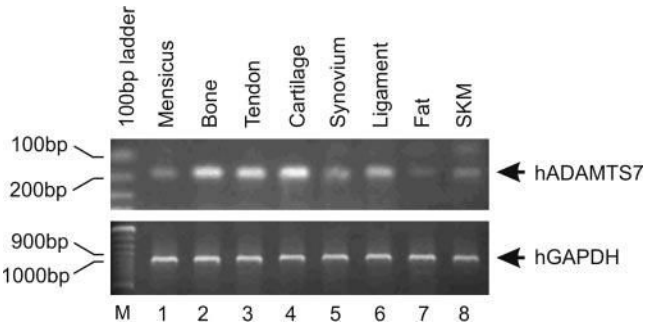

Figure 1.

ADAMTS-7 is expressed in human musculoskeletal tissues. Amplification products are consistent with a predicted size of 167 bp for hADAMTS-7 and 983 bp for hGAPDH. SKM = skeletal muscle; Lanes marked “M” contain DNA marker (Amersham Pharmacia Biotech, Piscataway, NJ).

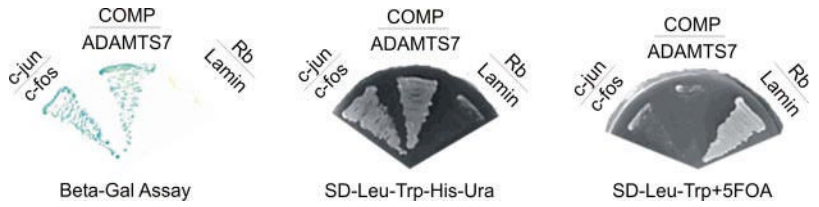

Figure 2.

Binding of COMP to ADAMTS-7 in assay to test 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 vector pPC86 (i.e., pPC86-c-jun, pPC86-ADAMTS-7, 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 sd- leu^-/trp^- plates and tested for β-galactosidase activity (*left panel*), for growth on plates lacking histidine and uracil and containing 3AT (*middle panel*, sd-$leu^-/trp^-/his^-/ura^-/3AT^+$), and for growth on plates containing 5-fluoroorotic acid (5FOA) (*right panel*, sd- $leu^-/trp^-/ 5FOA^+$). The known interaction between c-Jun and c-Fos is used as a positive control, and the lack of interaction between Rb and lamin serves as a negative control.

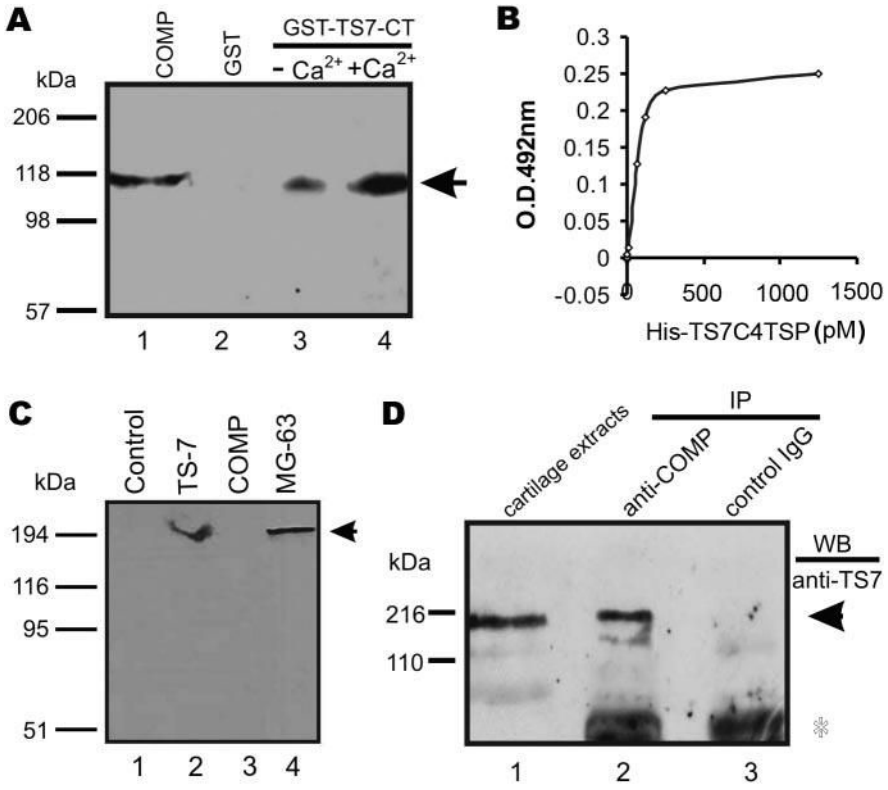

Figure 3.

COMP associates with ADAMTS-7 both *in vitro* and *in vivo*. *A*) GST pulldown assay. Purified GST (lane 2) or GST-TS7-CT fusion protein (lane 3 and 4) immobilized on GSH-Sepharose beads were incubated with purified hCOMP in the presence (lane 4) or absence (lane 3) of 5 mM Ca^{2+}. Proteins trapped by C terminal of ADAMTS-7 fused to GST were examined by immunoblotting with anti-COMP antibodies. Purified COMP (lane 1) was used as a positive control. Arrow indicates full-length COMP band. *B*) Solid-phase assay. Various amounts of recombinant His tagged C-terminal 4 TSP motifs of ADAMTS7 (His-TS7C4TSP) were immobilized on solid-phase 96-well microtiter plates. After being blocked, 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 liquid phase was detected using monoclonal antibodies to the bound COMP. *C*) Characterization of anti-ADAMTS-7 Ab. Cell lysates prepared from Sf9 insect cells infected with control (lane 1) or ADAMTS-7 bacluovirus (lane 2), from HEK293 cells stably transfected with a COMP expression construct (lane 3), and from MG-63 osteoblastic cells (lane 4) were subjected to SDS-PAGE and immunoblotted with affinity-purified anti-ADAMTS-7 antibodies. *D*) CO-IP assay. Cartilage extracts were incubated with either anti-COMP (lane 2) antiserum or control IgG (lane 3), followed by protein A/G agarose. Immunoprecipitated protein complex and cartilage extracts (lane 1, which provides a positive control) were examined by Western blotting with an anti-ADAMTS-7 Ab.

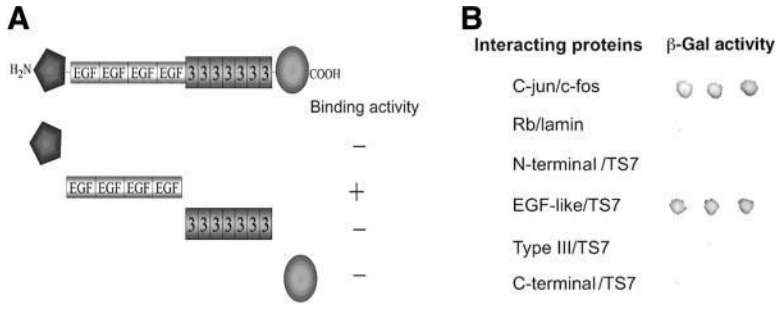

Figure 4.

ADAMTS-7 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-7. Presence or absence of binding between COMP domains and ADAMTS-7 is indicated a "+" or "-," respectively. *B*) β-Galactosidase activity was used to test interaction between the C-terminal domain of rADAMTS-7 and 1 of 4 domains of COMP. Three independent yeast transformants for each pair of plasmids were transferred onto a nitrocellulose membrane, and β-galactosidase activity was determined. The known interaction between c-Jun and c-Fos was used as a positive control, and the lack of interaction between Rb and lamin served as a negative control[b].

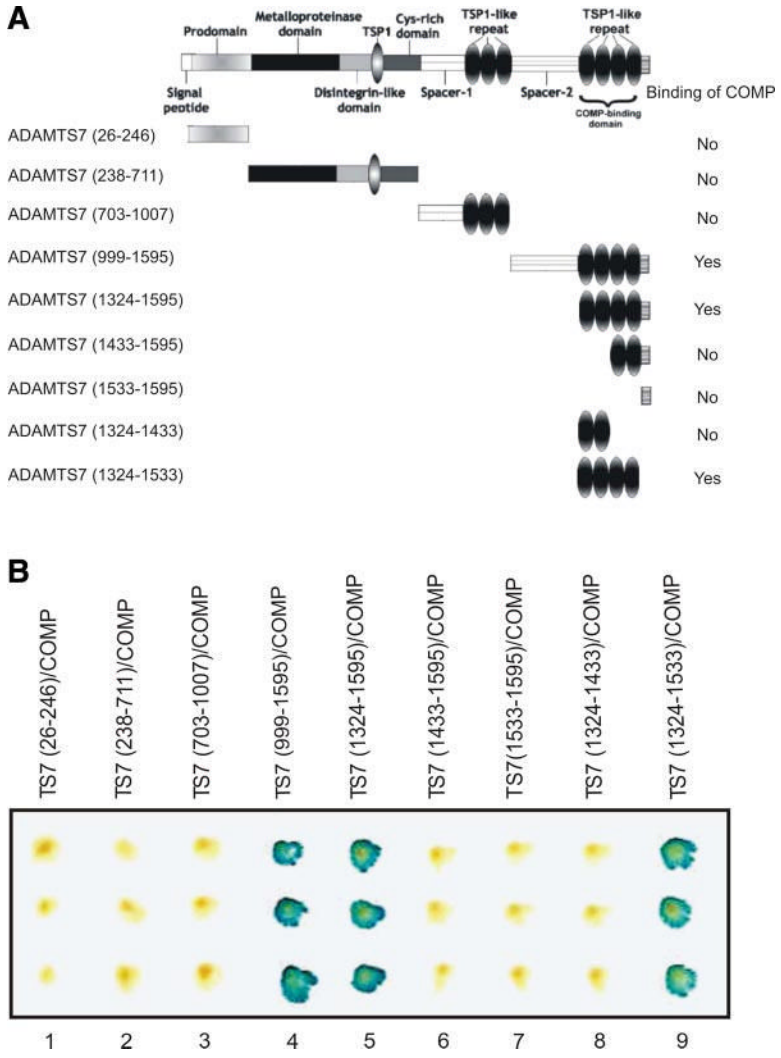

Figure 5.

Four C-terminal TSP motifs are required and sufficient for interaction with COMP. *A*) Schematic diagram of ADAMTS-7 constructs used to map those of its fragments that bind to COMP. Numbers refer to amino acid residues in the ADAMTS-7; ovals = TSP motifs. Interactions between COMP and ADAMTS-7 derivatives are summarized and indicated by "Yes" or "No." *B*) β-Galactosidase activity was used to test interaction between ADAMTS-7 derivatives and COMP. Three independent yeast transformants for each pair of plasmids were transferred onto a nitrocellulose membrane and the β-galactosidase activity determined.

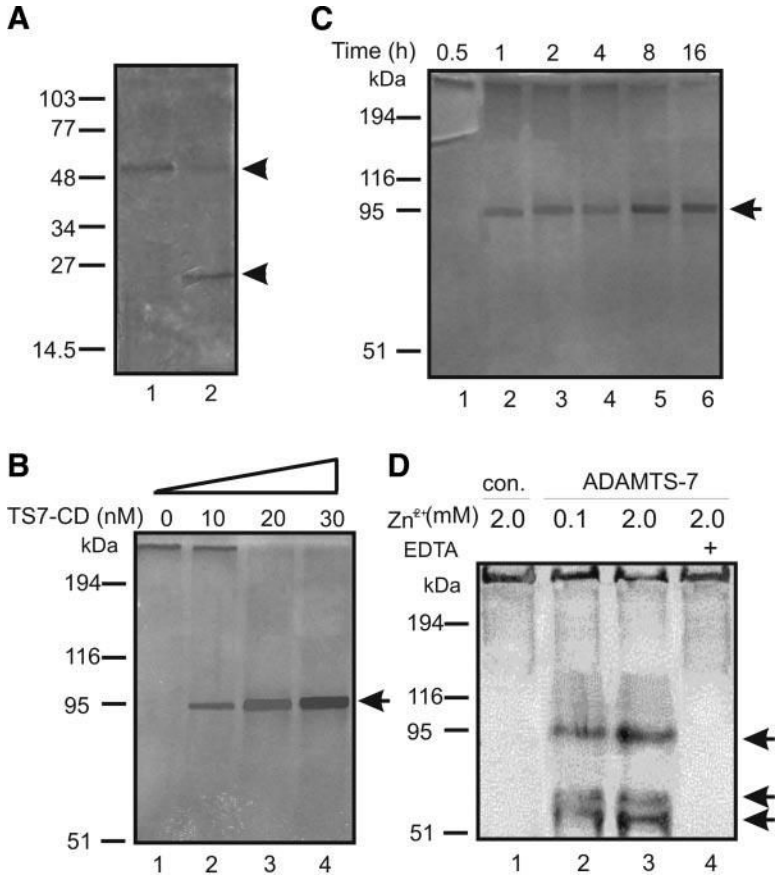

Figure 6.

ADAMTS-7 digests COMP *in vitro*. *A*) Purified GST-TS7-CD fusion protein (lane 1) and TS-7-CD without a GST moiety (lane 2) were separated by SDS-PAGE and visualized with silver staining. *B*) Catalytic domain of ADAMTS-7 digests COMP in a dose-dependent manner. Purified hCOMP was incubated with various amounts of catalytic domain of ADAMTS-7 (TS7-CD), as indicated; cleaved products were separated by nonreduced SDS-PAGE, and intact COMP and fragments were visualized with Coomassie brilliant blue G-colloidal solution. Resultant fragment is indicated by arrow. *C*) Catalytic domain of ADAMTS-7 digests COMP in a time-dependent manner. Purified hCOMP (200 nM) was incubated with recombinant catalytic domain of ADAMTS-7 (TS7-CD, 30 nM), and resultant products were analyzed as in *B. D*) Recombinant full-length ADAMTS-7 digests COMP *in vitro*. Purified COMP (200 nM) was incubated with the cell lystes prepared from Sf9 insect cells infected with either control (lane 1) or ADAMTS-7 bacluovirus in digestion buffer in the presence of indicated amounts of Zn^{2+} or 5 mM EDTA (lanes 2, 3, and 4), and nonreduced cleaved products were resolved by SDS-PAGE and detected by Western blotting with anti-COMP polyclonal antiserum.

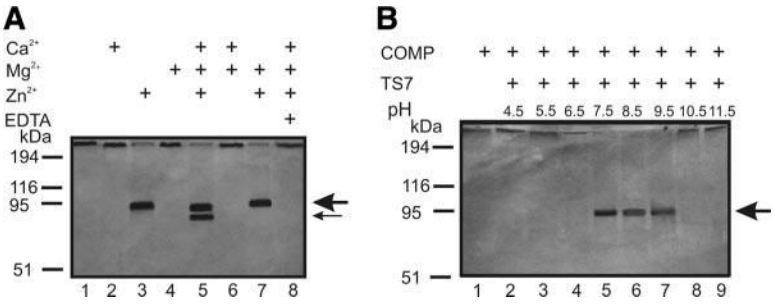

Figure 7.

Zn^{2+}- and pH-dependence of ADAMTS-7-mediated COMP digestion. *A*) Zn^{2+} is essential for the enzymatic activity of ADAMTS-7. Purified COMP (250 nM) was incubated with purified catalytic domain of ADAMTS-7 (25 nM) in digestion buffer supplemented with 5 mM $CaCl_2$, 2 mM $ZnCl_2$, 2.5 mM $MgCl_2$, or a combination of these or in the presence of 5 mM EDTA, as indicated; the digested proteins were resolved by 10% nonreduced SDS-PAGE and the gel was stained with Coomassie brilliant blue G-colloidal solution. Resultant fragment is indicated by large arrow; small arrow indicates additional (lower) band in lane 5, which is probably due to the conformation change in the presence of Ca^{2+}. *B*) Activity of ADAMTS-7 is pH dependent. The same digestion was performed in a buffer supplemented with 5 mM $CaCl_2$, 2 mM $ZnCl_2$, and 2.5 mM $MgCl_2$ at various pH, as indicated; digested proteins were resolved as in *A*.

Figure 8.

Increased expression of ADAMTS-7 in cartilage and synovium of RA patients. *A*) Expression of ADAMTS-7 in normal, OA, and RA cartilage assayed by real-time PCR; expression of ADAMTS-7 in each sample was normalized against 18S rRNA endogenous control. Normalized values were then calibrated against the normal cartilage value. *B*) Expression of ADAMTS-7 in normal and RA synovium. Samples were processed and data analyzed as in *A*. Units are arbitrary, and leftmost bar in each panel indicates a relative concentration of ADAMTS-7 of 1. $^{***}P < 0.001$ vs. N control.

TABLE 1

Fragments of rat ADAMTS-7 cloned into pPC86 vector

Fragment	Plasmid
Prodomain (a.a. 26-246)	pADAMTS-7 (26-246)
Metalloproteinase, disintegrin-like, and cysteine-rich domains (a.a. 238-711)	pADAMTS-7 (238-711)
Spacer-1 plus three middle TSP repeats (a.a. 703-1007)	pADAMTS-7 (703-1007)
Spacer-2 plus four C-terminal TSP repeats plus C-terminal unique region (a.a. 999-1595)	pADAMTS-7 (999-1595)
Four C-terminal TSP repeats plus C-terminal unique region (a.a. 1324-1595)	pADAMTS-7(1324-1595)
Two C-terminal TSP repeats plus C-terminal unique region (a.a. 1433-1595)	pADAMTS-7(1433-1595)
C-terminal unique region (a.a. 1533-1595)	pADAMTS-7(1533-1595)
Two C-terminal TSP repeats (a.a. 1324-1433)	pADAMTS-7(1324-1433)
Four C-terminal TSP repeats (a.a. 1324-1533)	pADAMTS-7(1324-1533)