Purification and Cloning of Aggrecanase-1: A Member of the ADAMTS Family of Proteins

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We purified, cloned, and expressed aggrecanase, a protease that is thought to be responsible for the degradation of cartilage aggrecan in arthritic diseases. Aggrecanase-1 [a disintegrin and metalloproteinase with thrombospondin motifs–4 (ADAMTS-4)] is a member of the ADAMTS protein family that cleaves aggrecan at the glutamic acid-373–alanine-374 bond. The identification of this protease provides a specific target for the development of therapeutics to prevent cartilage degradation in arthritis.

Aggrecan degradation is an important factor in the erosion of articular cartilage in arthritic diseases. This degradation involves proteolysis of the aggrecan core protein near the NH_2-terminus, where two major cleavage sites have been identified: One site is between Asn^{341} and Phe^{342}, where matrix metalloproteinases (MMPs) clip aggrecan (*1*), and the other site is between Glu^{373} and Ala^{374}, which is attributed to "aggrecanase" (*2*). Aggrecan fragments cleaved at the Glu^{373}-Ala^{374} bond have been identified in cultures undergoing cartilage matrix degradation (*3, 4*) and in arthritic synovial fluids (*5*). Some, but not all, MMP inhibitors were found to prevent aggrecan degradation, and this inhibition correlated with their potency against aggrecanase and not with their potency against MMPs (*4, 6*). The protease responsible for cleavage at the Glu^{373}-Ala^{374} bond has not yet been identified. We report the purification of aggrecanase-1 (ADAMTS-4), a member of the ADAMTS family of proteins, and the cloning of its cDNA.

Aggrecanase-1 was purified from interleukin-1 (IL-1)–stimulated bovine nasal cartilage (BNC)–conditioned media (*6*) by following its activity with an assay using the neoepitope antibody, BC-3 (*7*), to detect products formed by specific cleavage at the Glu^{373}-Ala^{374} bond. Purification included fractionation by chromatography on Macro S and gelatin-agarose columns, followed by affinity purification with (i) bovine tissue inhibitor of metalloproteinases–1 (TIMP-1), which inhibits aggrecanase activity with a median inhibitory concentration (IC_{50}) of 210 nM (*6*), and (ii) an aggrecanase-inhibitor resin (*8*). After the affinity steps, SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed one predominant protein, aggrecanase-1, that ran as a doublet at ~62 kD (Fig. 1A). An excised region of the gel containing the aggrecanase-1 protein exhibited activity, whereas that of the other regions of the gel did not (Fig. 1B). Inclusion of SF775 {(2*S*,11*S*,12*S*)-12-isobutyl-2-[(methylamino)carbonyl]-11-[(hydroxylamino)carbonyl]-8,13-dioxo-1,7-diazacyclotridecane)}, a potent aggrecanase inhibitor, blocked binding of the aggrecanase-1 doublet to the inhibitor resin (Fig. 1A), confirming that this protein represented aggrecanase.

Incubation of purified aggrecanase-1 with bovine aggrecan produced fragments generated by cleavage at the Glu^{373}-Ala^{374} bond (Fig. 1C). The pattern of BC-3–reactive bands produced was very similar to that generated upon the cleavage of cartilage aggrecan by endogenously generated aggrecanase in BNC stimulated with IL-1 (*4, 6*).

NH_2-terminal sequence analysis indicated that both bands of the aggrecanase-1 doublet represented the same protein, and the sequence of the first 27 residues was determined. In addition, two internal fragments, a 7- and an 11-mer, from a tryptic digest were identified. Homology searches of protein and expressed sequence tag (EST) databases found that these sequences were unique. A region of the aggrecanase-1 NH_2-terminus showed similarity to the NH_2-terminus of the catalytic domain of members of the adamalysin family of metalloproteinases, including atrolysin C, atrolysin D, ruberlysin, adamalysin II, and Lachesis hemorrhagic factor–II (LHF-II), suggesting that aggrecanase-1 might be related to this family of enzymes. In addition, both the NH_2-terminal and two internal sequences of bovine aggrecanase-1 were found to be 50 to 60% identical to the inflammation-associated murine protein, ADAMTS-1 (*9*), again suggesting that aggrecanase-1 is a member of the adamalysin or a disintegrin and metalloproteinase (ADAM) family of proteins.

The internal 7- and 11-mer peptide sequences of aggrecanase-1 were 100 and 91% (10 of 11 amino acids) identical, respectively, to sequences encoded by a murine EST 474985 (GenBank accession number AA041973). Sequences from the corresponding human cDNA were cloned by polymerase chain reaction (PCR) with primers designed from this murine EST (*10*). In addition, the partial cDNA, corresponding to murine EST 474985, was obtained and sequenced in its entirety. A search of the EST database with the resulting murine sequence identified a single human EST containing sequences from the 3′ untranslated region of the gene. Primers were designed from the initial human PCR product and the human EST, and a full-length open reading frame (ORF) was assembled. The cDNA sequence contains a 2511–base pair ORF encoding 837 amino acids (Fig. 2A).

The sequences of the three peptides derived from the purified bovine aggrecanase-1 are encoded within the human cDNA. A cloned portion of the bovine aggrecanase-1 cDNA, spanning nucleotides 679 to 2257, is 94% homologous to the human cDNA (*11*). The deduced amino acid sequence of this fragment shares 90% homology with the human sequence, confirming that we have indeed cloned the human ortholog of bovine aggrecanase-1.

As predicted on the basis of sequences from the purified bovine protein, aggrecanase-1 (Fig. 2A) is related to the adamalysin family. Aggrecanase-1 contains a signal sequence followed by a propeptide domain with a probable cysteine switch (*12*) at Cys^{194} and a potential furin cleavage site (*13*) (residues 208 to 215) that precedes the catalytic domain. The catalytic domain has a zinc-binding motif similar to the HEXXHXXGXXH (*14*) motif found in MMPs and ADAMs, with the exception that Asn^{368} is found in place of the glycine. An aspartic acid follows the third conserved histidine (Asp^{372}), as is found in the adamalysin family. Aggrecanase-1 also contains a disintegrinlike domain but lacks

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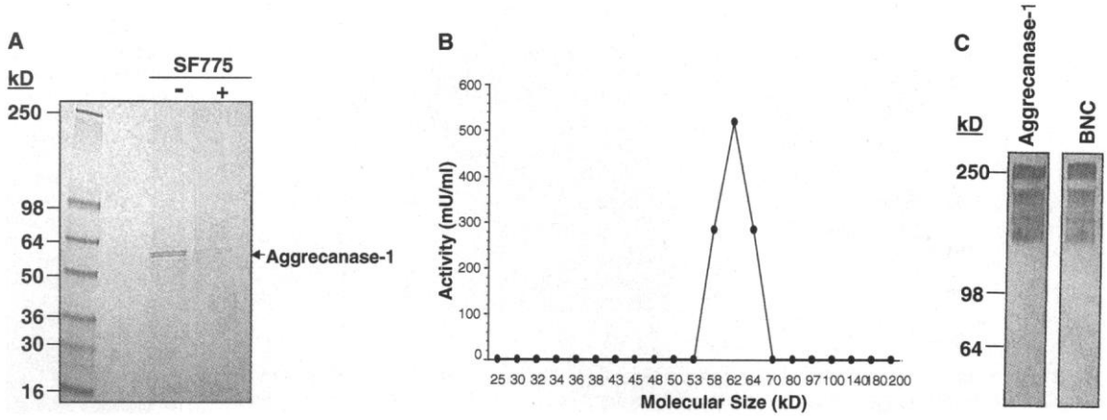

Fig. 1. Analysis of affinity-purified aggrecanase-1. (**A**) Coomassie blue–stained SDS-PAGE blot of affinity purified bovine aggrecanase-1. (**B**) Aggrecanase activity (*6*) in slices of SDS-PAGE nonreducing gel after electrophoresis and elution from gel. (**C**) Pattern of BC-3–reactive aggrecan fragments generated by the digestion of bovine aggrecan monomers with purified bovine aggrecanase-1 or in IL-1–stimulated BNC.

the transmembrane domain and cytoplasmic tail present in many adamalysins. Instead, aggrecanase-1 ends with a COOH-terminal domain that contains a thrombospondin (Tsp) type I motif (residues 521 to 575) that is similar to those present in ADAMTS-1.

The presence of a probable cysteine-switch sequence in aggrecanase-1 suggests that, like the MMPs, it is synthesized as a zymogen and is cleaved to remove the propeptide domain and generate the mature active enzyme. This is supported by data showing that a compound that interferes with normal pro-MMP activation through a cysteine-switch mechanism (*15*) inhibits IL-1–induced cleavage of aggrecan at the Glu^{373}-Ala^{374} bond in cartilage organ cultures (*4*).

Although the disintegrinlike domain of aggrecanase-1 is relatively well-conserved, its function is unclear. However, it may serve to interact with integrins expressed on the chondrocyte cell surface (*16*). This would be consistent with observations that a loss of aggrecan from cartilage appears to occur initially around the chondrocytes.

Tsp type I repeats have been implicated in the interaction of Tsp with sulfated glycosaminoglycans (*17*). In addition, recent data indicate that the Tsp motif and submotifs of murine ADAMTS-1 bind to heparin and the extracellular matrix (*9, 18*). Thus, this region may serve to bind aggrecanase-1 to the glycosaminoglycans of the aggrecan substrate. Deglycosylation of aggrecan decreases the ability of aggrecanase to generate fragments formed by cleavage at the Glu^{373}-Ala^{374} bond (*19*), which is consistent with this hypothesis.

A recombinant form of the human aggrecanase-1 protein was generated corresponding to amino acids Met^{1} to Lys^{837}. Analysis by SDS-PAGE and protein immunoblotting (*20*) (Fig. 3A) identified a predominant band of ~64 kD. NH_2-terminal sequencing of this protein showed that the NH_2-terminus began with Phe^{213}, indicating that the prodomain was removed by cleavage at the Arg^{212}-Phe^{213} bond within the furin-sensitive sequence to produce the mature form of the protease. The mature form was found in media from IL-1–stimulated BNC, indicating that the proenzyme is similarly processed in mammalian cells.

NH_2-terminal sequence analysis of products from activity assays confirmed that human aggrecanase-1 cleaved bovine aggrecan as expected between Glu^{373} and Ala^{374} and not at the Asn^{341}-Phe^{342} bond, where MMPs preferentially cleave this substrate (*21*). Cleavage produced a BC-3–reactive fragment pattern (Fig. 3B), indistinguishable from that generated by purified bovine aggrecanase-1 and very similar to that produced by aggrecanase activity generated in situ in cartilage cultures stimulated with IL-1 (Fig. 1C). In addition, we found that aggrecanase-1 is ineffective in cleaving several substrates cleaved by MMPs, including the extracellular matrix molecules, type II collagen, Tsp, and fibronectin, as well as the more general protease substrates, casein and gelatin (*21*).

Inhibitor studies showed that human aggrecanase-1 activity was inhibited by several hydroxamates that are effective in blocking the cleavage of aggrecan at the Glu^{373}-Ala^{374} bond by native bovine aggrecanase. In addition, the rank order of potency of the inhibitors was the same against bovine aggrecanase and recombinant human aggrecanase-1 (Table 1).

The sequence for aggrecanase-1 was recently deposited as GenBank accession number KIAA0688, an unidentified human gene from a set of size-fractionated human brain

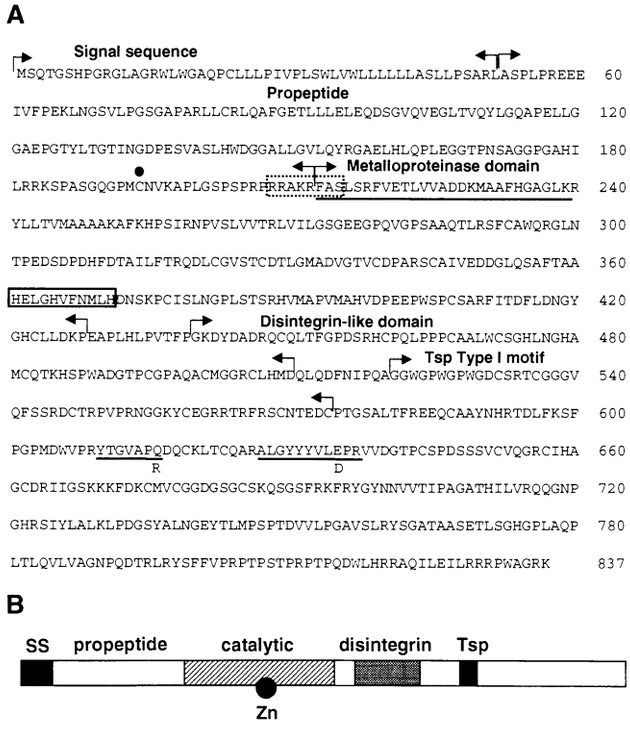

Fig. 2. Deduced sequence of human aggrecanase-1. (**A**) Amino acid sequence (*14*) deduced from the aggrecanase-1 cDNA. Domains are labeled above the sequence and delineated by arrows. Underlined sequences correspond to the NH_2-terminal peptide and internal peptide sequences for the purified bovine protein; residues that are different in the bovine sequence are shown below the underline. The conserved zinc-binding motif (solid line) and furin-sensitive sequence (dashed line) are boxed. Solid circle denotes the location of Cys^{194} involved in a probable cysteine switch. The nucleotide sequence was deposited with GenBank (accession number AF148213). (**B**) Diagram of the domain structure of aggrecanase-1. The protein consists of a signal sequence (SS), a propeptide domain, a catalytic domain, a disintegrin-like domain, and a COOH-terminal domain with a Tsp type I motif.

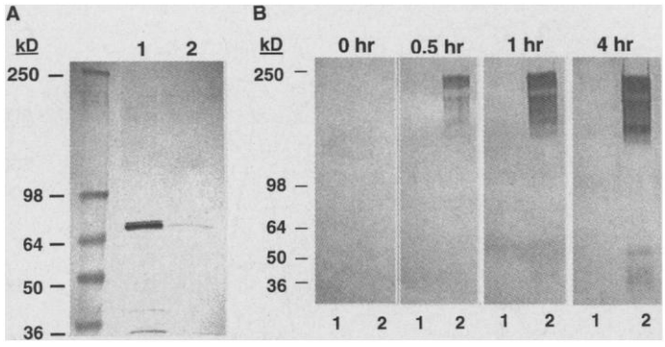

Fig. 3. Expression of recombinant human aggrecanase-1. (**A**) Protein immunoblot analysis of media samples from human aggrecanase-1–transfected *Drosophila* S2 cells. Lane 1, media from cells 24 hours after induction; lane 2, media from uninduced cells. (**B**) Assay of enzymatic activity in media from human aggrecanase-1–transfected *Drosophila* S2 cells. Media were incubated for 0, 0.5, 1, and 4 hours with bovine aggrecan followed by evaluation of fragments by BC-3 protein immunoblot analysis. Lane 1, media from uninduced cells; lane 2, media 24 hours after induction.

cDNA libraries (*22*). Our evaluation of RNA from multiple tissues by Northern blot analysis or reverse transcriptase–PCR (RT-PCR) indicates that the aggrecanase-1 message is present in brain tissues as well as lung and heart tissues, with very low levels in placenta and skeletal muscle tissues (*23*). We also observed up-regulation of the aggrecanase-1 message in stimulated human fetal chondrocytes and in joint tissues from adjuvant arthritic rats by RT-PCR (*11*).

Using a different purification scheme, we identified a second aggrecanase, aggrecanase-2, with a similar specificity for the cleavage of aggrecan at the Glu^{373}-Ala^{374} bond (*23*). In contrast, our preliminary data indicate that ADAMTS-1 does not cleave aggrecan at the Glu^{373}-Ala^{374} bond (*24*). Ongoing studies suggest that there may be additional family members. However, the identity of these proteins and whether they will display aggrecanase enzymatic activity have not yet been determined.

In the work reported herein, we purified the metalloproteinase, aggrecanase-1 (ADAMTS-4), from IL-1–stimulated BNC and then cloned and expressed its human ortholog. This protease represents a cartilage aggrecanase that cleaves aggrecan at the Glu^{373}-Ala^{374} bond to produce fragments similar to those found in the synovial fluid of patients with various types of arthritic diseases. The identification of this enzyme and production of recombinant human aggrecanase-1 provide a target for the development of therapeutics to prevent the loss of articular cartilage in arthritis.

Table 1. Inhibitor potency against native bovine aggrecanase and against recombinant human aggrecanase-1. Bovine aggrecan was incubated with native bovine aggrecanase in cartilage-conditioned media or with recombinant human aggrecanase-1 in the absence or presence of inhibitor and products monitored by BC-3 protein immunoblot analysis (*6*).

Inhibitor	Bovine aggrecanase IC_{50} (nM)	Recombinant human aggrecanase-1 IC_{50} (nM)
SE206	137	76
BB-16	548	159
XS309	>10,000	2185

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8. Conditioned media from IL-1–stimulated BNC (*6*) was supplemented with 1 μM leupeptin, 1 μM pepstatin, 1 mM PMSF, and 0.05% (v/v) Brij-35; filtered; and loaded onto a Macro S column. The column was washed with buffer A [50 mM Hepes (pH 7.5), 10 mM $CaCl_2$, 0.1 M NaCl, and 0.05% Brij-35], and aggrecanase activity was eluted with buffer A containing 1.0 M NaCl. The elute was supplemented with the MMP inhibitor XS309 {*N*,3-methyl-(3*R*)-2-[(2*S*)-2-[(1*R*)-2-(hydroxyamino)-1-methyl-2-oxoethyl]-4-methylpentanoyl]hexahydro-3-pyridazinecarboxamide} and loaded onto a gelatin-agarose column. Material passing through this column, which contained the aggrecanase activity, was incubated with 1 μM bovine TIMP-1 (Oncogene, Cambridge, MA), followed by incubation with a TIMP-1 monoclonal antibody [PF020, immunoglobulin G (IgG), Oncogene] at a 1:5000 dilution. The TIMP-1–antibody complex was then applied to a protein A column that was washed three times with buffer B [10 mM tris (pH 7.5), 250 mM NaCl, and 0.025% Tween20], and the protein was eluted with 100 mM glycine/HCl (pH 2.5). The eluate was neutralized with 1 M tris base and incubated with an aggrecanase-inhibitor resin. XS309 was included during affinity purification to retard the binding of MMPs to the TIMP-1 and the inhibitor resin. The resin was washed at 4°C with buffer B, and bound aggrecanase was eluted with 4 M guanidine HCl. The eluate was dialyzed and run on SDS-PAGE (10 to 20% acrylamide), and protein was detected by Coomassie blue staining. The protein was immobilized on polyvinylidene difluoride (PVDF) and subjected to NH_2-terminal sequence analysis. Elute samples were separated by SDS-PAGE under nonreducing conditions, and the aggrecanase-1 protein was excised along with multiple slices of the same gel that did not contain any detectable protein. Gel slices were incubated with 1% Triton X-100; crushed in 50 mM tris (pH 7.5), 100 mM NaCl, and 10 mM $CaCl_2$; dialyzed; and analyzed for aggrecanase activity (*6*).
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10. Sequences were amplified from human heart cDNA (Clontech, Palo Alto, CA) with PCR primers designed from murine EST 474985 (GGGGGTGGTGTCCAGTTCTCC and GGCCCTGGAAAGCTCTTGAAGAG). Primers were designed from the resulting PCR product (CCAGTTGGGCAGTCCTCAGTGTT and GGTCGGTGCGGTGGTTGTAGGC) and were used to amplify a 2.2-kb 5′ rapid amplification of cDNA ends (RACE) product from heart cDNA with the Marathon cDNA Amplification System (Clontech). In addition, the IMAGE consortium [G. Lennon, C. Auffray, M. Polymeropoulos, M. B. Soares, *Genomics* **33**, 151 (1996)] clone corresponding to EST 474985 was sequenced in its entirety and used to identify a human EST (GenBank accession number D45652) containing sequences from the 3′ untranslated region of the gene. Primers designed from the human PCR product (CCCCGGAATGGTGGCAAGTACTG) and from the human EST (ACCCACATCTGTCTGACTCCAAA) were used to amplify sequences from the 3′ end of the transcript from human heart cDNA. A full-length ORF was assembled with a 5′ RACE clone and a 3′ fragment obtained by RT-PCR. Expression vectors containing the full-length ORF were assembled for subcloning into the pRMHA3 [T. A. Bunch, Y. Grinblat, L. S. Goldstein, *Nucleic Acids Res.* **16**, 1043 (1988)] for *Drosophila* S2 expression as described [D. C. Rio and G. M. Rubin, *Mol. Cell Biol.* **5**, 1833 (1985)].
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14. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr.
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20. Samples were separated by SDS-PAGE with 4 to 12% tris glycine gels, transferred to PVDF membranes, and probed with a rabbit polyclonal antibody to peptide, which recognizes the sequence VMAHVDPEEP (*14*) (residues 393 to 403 in Fig. 2A). The membranes were incubated with goat antimouse IgG alkaline phosphatase conjugate and visualized by incubation with NBT/BCIP substrate.
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