The Interglobular Domain of Cartilage Aggrecan Is Cleaved by Hemorrhagic Metalloproteinase HT-d (Atrolysin C) at the Matrix Metalloproteinase and Aggrecanase Sites*

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Two primary cleavage sites have been identified within the interglobular domain of the cartilage aggrecan core protein: one is between amino acid residues Asn341 and Phe342, where many matrix metalloproteinases (MMP) have been shown to cleave; and the other is between amino acid residues Glu373 and Ala374. Although cleavage at the Glu373-Ala374 site is believed to play a critical role in cartilage aggrecan degradation in arthritic diseases, the enzyme responsible for cleavage at this site, "aggrecanase," has not been identified. Members of the ADAM (α disintegrin and metalloproteinase) family of proteins, which shows structural homology to the snake venom hemorrhagic metalloproteinases (reprolysins), have recently been demonstrated to be expressed in articular chondrocytes. Because many ADAM family members have a putative proteinase function, this raises the possibility that aggrecanase may be a member of this family of proteases. To examine whether reprolysins have the ability to cleave aggrecan at either the aggrecanase site or the MMP site, the snake venom hemorrhagic toxin metalloproteinase HT-d (atrolysin C) was tested for its ability to cleave bovine aggrecan monomer. Cleavage was monitored using the BC-3 antibody, which recognizes aggrecan fragments with the new NH2 terminus ARGSV generated by cleavage at the aggrecanase site, and with the AF-28 antibody, which recognizes aggrecan fragments with the new NH2 terminus FFGVG generated by cleavage at the MMP site. Cleavage at both the aggrecanase and MMP sites occurred in a concentration-dependent manner with 100 nM atrolysin C or greater. AF-28-reactive fragments were generated by 30 min of incubation, and levels were maximal by 8 h; BC-3-reactive fragments were detected at 2 h and continued to increase through 48 h, thus suggesting that atrolysin C can cleave at the MMP and aggrecanase sites. NH2-terminal aggrecan fragments generated by cleavage at the aggrecanase site were also detected using antisera recognizing the new COOH terminus, NITEGE, formed by cleavage at the Glu373-Ala374 bond, indicating that cleavage at this site does not require prior cleavage at the MMP site. These data provide the first demonstration that a reprolyn can cleave the core protein of aggrecan and the first example of a specific protease that can cleave at the aggrecanase site independent of cleavage at the MMP cleavage site.

Aggrecan is the major proteoglycan of cartilage and provides this tissue with its mechanical properties of compressibility and elasticity. Aggrecan monomers interact with hyaluronic acid (HA) and are usually found as part of a large aggregate containing 10–100 monomers/HA molecule. The primary role of aggrecan is to swell and hydrate the framework of cartilage collagen fibrils thus providing cartilage with its properties of compressibility and elasticity. The NH2 terminus of the aggrecan monomer core protein is comprised of two globular domains called G1 and G2 which are separated by an interglobular domain that spans about 150 residues in length. The G2 region is followed by a long central glycosaminoglycan attachment region and a COOH-terminal globular domain, G3 (1, 2).

In cartilage degradation associated with diseases such as osteoarthritis and rheumatoid arthritis, aggrecan is one of the first matrix components to undergo measurable loss, which ultimately leads to loss of cartilage function. Proteolytic cleavage within the interglobular domain is believed to be responsible for the loss of aggrecan from cartilage. However, the enzyme responsible for aggrecan cleavage in these diseases has yet to be identified.

Two major sites of proteolytic cleavage have been identified within the interglobular domain, one between amino acids Asn341 and Phe342 and the other between Glu373 and Ala374. Many matrix metalloproteinases, including MMP-1, -2, -3, -7, -8, -9, and -13, have been shown to cleave aggrecan at the Asn341-Phe342 site, but to date attempts to generate cleavage at the Glu373-Ala374 site with a number of MMPs and other purified proteases have been relatively unsuccessful (3–7). Of the enzymes evaluated, only MMP-8 has shown the ability to clep at this site (8, 9), and cleavage was seen only after cleavage at the Asn341-Phe342 MMP site. Thus, cleavage at the Glu373-Ala374 site has been attributed to an as yet unidentified enzyme given the name “aggrecanase” because of its ability to cleave the aggrecan core molecule.

A novel family of metalloproteinases named ADAMs (α disintegrin and metalloproteinase) has been described (10–14). These proteins are structurally related to the snake venom hemorrhagic toxins (reprolysins) and contain minimally a metalloproteinase domain, and some members have additional domain structures such as NH2-terminal propeptide domains, disintegrin domains, and cysteine-rich domains. Cell-associated ADAMs also contain an epidermal growth factor-like transmembrane domain and cytoplasmic domain at the COOH terminus of the molecule. Recent studies have demonstrated that human articular chondrocytes express mRNA for three members of the ADAM family, ADAM-10, -12, and -15 (15). In addition, some ADAM proteins have been shown to be up-

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§ The abbreviations used are: HA, hyaluronic acid; MMP, matrix metalloproteinase; bHA, biotinylated hyaluronic acid.

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regulated at the mRNA level by interleukin-1 (16), suggesting that their expression may be inflammation-associated. Members of the repolysin family of enzymes have been shown to degrade basement membranes and cleave the extracellular matrix molecules, type IV collagen, laminin, and fibronectin (17). X-ray crystal structures of repolysins show that their structure is similar to that of the astacin family and the MMPs, especially within the active site region (18). Taken together these data open the possibility that ADAM family members may be involved in the proteolytic cleavage of the cartilage extracellular matrix.

In this study we investigated the ability of a repolysin, atrolysin C, to cleave the aggrecan core protein within the interglobular domain. Using the monoclonal antibody BC-3, which recognizes the NH$_2$-terminal neoepitope ARGSV, generated by cleavage at the Glu$_{373}$-Ala$_{374}$ aggrecanase site (19), and the monoclonal antibody AF-28, which recognizes the NH$_2$-terminal neoepitope FFGVG, generated by cleavage at the Asn$_{341}$-Phe$_{342}$ MMP site (20) (Fig. 1), we demonstrate that atrolysin C is capable of cleaving cartilage aggrecan at both the aggrecanase site and the MMP site. Our results also show that these cleavages occur concomitantly, unlike cleavage by MMP-8, which required complete depletion of the preferred Asn$_{341}$-Phe$_{342}$ MMP site to clip at the aggrecanase site (8, 9). These findings establish that a repolysin is able to cleave aggrecan at the aggrecanase site and open the possibility that the as yet unidentified cartilage aggrecanase enzyme may be a member of the ADAM family of proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—The snake venom hemorrhagic toxin HT-d (atrolysin C) was purified from rattlesnake venom (21). Purity of the atrolysin C was demonstrated by silver stain analysis where only one band was detected after loading 100 ng of total protein on a 10% gel. The activity of the atrolysin C sample could be blocked completely by the metalloproteinase inhibitor EDTA and by a specific hydroxamate MMP inhibitor. Chondroitinase ABC lyase (Proteus vulgaris) (EC 4.2.2.4), keratanase (Pseudomonas sp.) (EC 3.2.1.103), and keratanase II (Bacillus sp.) were from Seikagaku (Kogyo, Japan). Polyvinylidine difluoride membrane filters used for Western transfer and 8–16% Tris-glycine gels used to separate aggrecan products were from Novex (San Diego, CA). Goat anti-mouse IgG alkaline phosphatase conjugate and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development used for Western detection were from Promega (Madison, WI). Streptavidin-alkaline phosphatase was purchased from Life Technologies, Inc.

**Aggrecan Isolation**—Nasal septa were removed from bovine noses obtained fresh from slaughter. The cartilage was sliced, and aggrecan was extracted by stirring at 4 °C for 48 h in 10 volumes of 4 M guanidine-$\text{HCl}$ in 0.05 M sodium acetate, pH 5.8, containing the protease inhibitors 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 2 mM phenylmethanesulfonyl fluoride, and 0.05 M benzamidine-$\text{HCl}$. Aggrecan monomers were isolated by equilibrium density gradient centrifugation in cesium chloride (24, 25), and the bottom of this gradient (d $> 1.54$ g/ml) containing the monomers was dialyzed at 4 °C against water, lyophilized, and stored at −20 °C.

**Enzyme Digestion**—Digestions were carried out in 100 μl of 50 mM Tris-$\text{HCl}$ buffer, pH 7.5, containing 100 mM NaCl and 10 mM CaCl$_2$. Purified bovine aggrecan (500 μg) was incubated with atrolysin C at 37 °C for the indicated times and concentrations. The reactions were quenched with EDTA and analyzed by labeling with bHA or by immunolocalization with MAB2005, BC-3, NITEGE, or AF-28 antibodies in a Western blot analysis.

**Analysis of Aggrecan Products**—For analysis of fragments by Western blot, aggrecan was enzymatically deglycosylated with chondroitinase ABC (0.1 unit/10 μg of aggrecan) for 1 h at 37 °C and then with keratanase (0.1 unit/10 μg of aggrecan) and keratanase II (0.002 unit/10 μg of aggrecan) for 2 h at 37 °C in buffer containing 50 mM sodium acetate, 0.1 M TriS-$\text{HCl}$, pH 6.5. After digestion the aggrecan was precipitated with 5 volumes of acetone and reconstituted in 30 μl of TriS-glycine-SDS sample buffer containing 2.5% β-mercaptoethanol and heated for 3 min at 100 °C. Samples to be analyzed in the bHA Western were not reduced and not heated before SDS-polyacrylamide gel electrophoresis to maintain the structural integrity of the G1 domain.

**Western Blot Analysis**—20 μg of glycosaminoglycan from each sample was loaded on an 8–16% TriS-glycine gel and separated by SDS-polyacrylamide gel electrophoresis under reducing conditions. The separated proteins were transferred to polyvinylidine difluoride membranes and immunolocalised with a 1:500 dilution of the BC-3 antibody, a 1:1000 dilution of the AF-28 antibody, a 1:5000 dilution of the NITEGE antibody, a 1:2,000 dilution of the MAB2005 antibody, or with 2 μg/ml bHA. Membranes that were analyzed in the bHA Western for a functional G1 domain were washed more thoroughly with Tri-buffered

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**Fig. 1. Aggrecanase and MMP cleavage sites in bovine aggrecan interglobular domain.** Intact aggrecan diagram indicating regions of the molecule recognized by biotinylated hyaluronic acid (bHA) and MAB2005 (A). Aggrecanase cleaves aggrecan between amino acids Glu$_{373}$ and Ala$_{374}$ to form a small G1 fragment with the new COOH terminus NITEGE and a large COOH-terminal fragment with the new NH$_2$ terminus ARGSV (B). MMPs cleave aggrecan between amino acids Asn$_{341}$ and Phe$_{342}$ to form a small G1 fragment with the new COOH terminus DIPEN and a large COOH-terminal fragment with the new NH$_2$ terminus FGGVG (C). The ARGSVIL epitope is detected by the BC-3 antibody, the NITEGE epitope is detected by the NITEGE antibody, and the FGGVG epitope is detected by the AF-28 antibody.

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**Atrolysin C Cleaves Aggrecan at the MMP and Aggrecanase Sites**
saline to remove any residual SDS to allow the aggrecan fragments to renature to their native conformation before the addition of bHA. Subsequently, the membranes were incubated with a 1:5,000 dilution of goat anti-mouse IgG alkaline phosphatase conjugate for the BC-3, AF-28, NITEGE, and MAB2005 analysis or a 1:8,000 dilution of streptavidin alkaline phosphatase conjugate for the bHA analysis, and the aggrecan cleavage products were visualized by developing the blots in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color developing reagent. Overnight transfer resulted in complete transfer of developing reagent. Refrigerated blots were dried and exposed to autoradiography film.

RESULTS

Aggrecan Cleavage by Atrolysin C—Atrolysin C (0.5 μM) cleaved aggrecan in a time-dependent manner at several sites, resulting in the generation of five chondroitin sulfate-containing fragments, as detected by CSPG Western analysis using the MAB2005 antibody (panel A) or by binding with bHA (panel B). Molecular mass markers are indicated with large arrows, and protein bands described are indicated by small arrows.

Cleavage at the Asn341-Phe342 MMP Site by Atrolysin C—Atrolysin C cleaved aggrecan at the Asn341-Phe342 MMP site. Purified aggrecan monomer was incubated with various concentrations of atrolysin C for 24 h at 37 °C (panel A) or with 0.5 μM atrolysin C for 0, 0.5, 1, 2, 4, 6, 8, 24, and 48 h (panel B), and specific cleavage at the Asn341-Phe342 MMP site was detected by Western analysis using the AF-28 antibody, indicating that these fragments lack the G1 region.

Aggrecan Cleavage at the Asn541-Phe542 MMP Site by Atrolysin C—Aggrecan at the Asn541-Phe542 MMP site (Fig. 1) in a concentration-dependent manner after a 24-h incubation, as detected by AF-28 Western analysis (Fig. 3A). Three AF-28-reactive bands were detected, a doublet at ~180 kDa and a single band at ~80 kDa. With as little as 0.1 μM atrolysin C, cleavage at the MMP site was observed, with total band intensity increasing through 0.5 μM. However, the banding pattern shifted with increasing enzyme concentration. The larger AF-28-reactive fragment of the 180 kDa doublet was no longer detected at concentrations of 0.5 μM or higher, whereas at 1 and 5 μM total band intensity decreased, suggesting that at higher concentrations these fragments were degraded further to smaller fragments that run off the gel.

Aggrecan incubated for various times with 0.5 μM atrolysin C was analyzed for cleavage by AF-28 Western blot analysis. Generation of low levels of AF-28-reactive fragments were detected in as little as 30 min of incubation (Fig. 3B). Total AF-28-reactive band intensity showed a time-dependent increase up to 8 h but then began to decrease at 24 h and continued to decrease through 48 h. As was seen with increasing enzyme concentration, the banding pattern also shifted with increasing incubation time. At 30 min and 60 min a triplet of AF-28-reactive bands was seen at 180 kDa. However, the largest AF-28 band of the 180 kDa triplet was no longer present at 2 h, and by 24 h the second largest band of the 180 kDa triplet was also no longer detected, suggesting further cleavage had occurred with time. An additional band was observed by 1–2 h at 80 kDa which increased in intensity over time. The 180 kDa AF-28-reactive fragment was detected by the MAB2005 antibody in the chondroitin sulfate proteoglycan Western analysis, indicating that the 180 kDa fragment contains a portion of the highly glycosylated region of the aggrecan molecule between the G2 and G3 domain and has the NH₂ terminus FFGVG. These data suggest that this fragment was derived by
cleavage at the MMP site and at a site within the chondroitin-sulfate-rich region between G2 and G3 which would yield a fragment of approximately this size. The 80 kDa AF-28-reactive fragment does not appear to react well with the MAB2005 antibody and thus does not likely contain a significant portion of the chondroitin sulfate-rich region. Thus the 80 kDa fragment is most likely derived from cleavage at the MMP site and a site near the G2 domain resulting in loss of the majority of the glycosaminoglycan-rich region of the molecule.

Aggrecan Cleavage at the Glu373-Ala374 Aggrecanase Site by Atrolysin C—Atrolysin C cleaved purified aggrecan monomer at the Glu373-Ala374 aggrecanase site (Fig. 1) in a concentration-related manner as detected by BC-3 Western analysis (Fig. 4A) after a 24-h incubation. Four BC-3-reactive bands were detected, a doublet at ~150 kDa, and a doublet at ~64 kDa. With as low as 100 nM atrolysin C, cleavage at the aggrecanase site was observed, with total band intensity increasing through 1 μM. However, with increasing enzyme concentration the intensity of the bands at 150 kDa decreased in intensity, suggesting that these larger fragments were cleaved further at the COOH terminus resulting in conversion to fragments represented by the 64 kDa doublet or to smaller fragments that ran off the gel.

Atrolysin C at a concentration of 0.5 μM cleaved aggrecan in a time-dependent manner at the Glu373-Ala374 bond as determined by BC-3 Western and NITEGE Western. The generation of BC-3-reactive fragments (Fig. 4B) was detected with as little as 2 h of incubation, and total band intensity continued to increase with time through 48 h. However, there was an alteration in the banding pattern with time. A 150 kDa doublet was detected at 2 h. The larger size band of the doublet decreased in intensity at 8 h and was no longer detected by 24 h, whereas the smaller size band of the doublet continued to increase in intensity with time. At 24 h a second doublet was detected at 64 kDa which increased in intensity at 48 h. The 150 kDa BC-3-reactive fragment appears to be detected by the MAB2005 antibody in the chondroitin sulfate proteoglycan Western analysis, indicating that this fragment contains a portion of the highly glycosylated region of the aggrecan molecule between the G2 and G3 domains and has the NH2 terminus ARGSV.

These data suggest that the 150 kDa fragment was derived by cleavage at the aggrecanase site and at a site within the region between the G2 and G3 domain. The 64 kDa BC-3-reactive fragment does not appear to react well with the MAB2005 antibody and thus does not likely contain the chondroitin sulfate-rich region between G2 and G3. These data suggest that the 64 kDa fragment is most likely derived from cleavage at the aggrecanase site and at a site near the G2 domain which would exclude the glycosaminoglycan-rich region and yield a fragment of this approximate size.

Aggrecan fragments reactive with the NITEGE antibody (Fig. 5) were observed with as little as a 30-min incubation with atrolysin C and increased in intensity in a time-dependent manner up to 24 h. The NITEGE-reactive fragments appeared as a doublet between 64 and 70 kDa, consistent with the G1-NITEGE products obtained previously after interleukin-1β treatment of bovine chondrocytes (26). Detection of fragments reactive with the NITEGE antiserum prior to those reactive with the BC-3 antiserum upon cleavage at the Glu373-Ala374 bond is most likely caused by the different affinities of these antibodies for their antigens.

**DISCUSSION**

In the work reported here, we demonstrate that atrolysin C at concentrations as low as 0.1 μM cleaves aggrecan at both the Asn341-Phe342 MMP site and at the Glu373-Ala374 aggrecanase site. The ability to cleave aggrecan between amino acids Glu373 and Ala374 makes atrolysin C the only enzyme to date, other than neutrophil collagenase (MMP-8), to be shown capable of cleaving aggrecan at the aggrecanase site (8, 9). We have demonstrated previously that recombinant human MMP-8 cleaves native aggrecan in freeze-thawed bovine nasal cartilage at the Glu373-Ala374 aggrecanase site at concentrations similar to those effective in these studies with atrolysin C. However, MMP-8 was found to cleave preferentially at the Asn341-Phe342 MMP site and only cleaved at the Glu373-Ala374 aggrecanase site when complete depletion of substrate containing the preferred cleavage site was achieved (9). In contrast, atrolysin C appears to cleave simultaneously at both the Glu373-Ala374 aggrecanase site and the Asn341-Phe342 MMP site. This is supported by the ability of atrolysin C to generate the G1-NITEGE species. Both the AF-28-reactive species generated by cleavage at the MMP site and the NITEGE-reactive species generated by cleavage at the aggrecanase site were observed after a 30-min incubation with atrolysin C. The appearance of the NITEGE epitope prior to the appearance of the ARGGSVIL
epitope, which is present on the COOH-terminal fragment generated by this cleavage, is most likely caused by the different affinities of the BC-3 and NITEGE antibodies for their respective antigens. Since the MMP site (Asn341-Phe342) is between the G1 at the NH2 terminus of the aggrecan core protein and the aggrecanase site (Glu373-Ala374), prior cleavage at the MMP site would preclude the generation of a G1-NITEGE species. Thus, cleavage at the aggrecanase site by atrolysin C could not require previous cleavage at the MMP site as is the case with MMP-8. Generation of all three species, ARGSVIL, FFGVVG, and NITEGE, by atrolysin C is only consistent with this protease independently clipping at the MMP and aggrecanase sites or clipping first at the aggrecanase site and then further processing some of the COOH-terminal fragments by cleavage at the MMP site. Cleavage of aggrecan by atrolysin C differs from that by aggrecanase generated in chondrocyte cultures stimulated with interleukin-1 or retinoic acid where aggrecanase is apparently capable of cleaving at the Glu373-Ala374 bond in the complete absence of any detectable cleavage at the MMP site (26).

The MAB2005 Western and bHA analysis suggest that atrolysin C first cleaves aggrecan between the G2 and G3 globular domains, to produce three large fragments with an intact G1 domain, a 215 kDa doublet and a 190 kDa band. These fragments are then processed further via cleavage within the interglobular domain at the MMP Asn341-Phe342 and aggrecanase Glu373-Ala374 sites to produce fragments that lack a G1 domain. Cleavage of aggrecan by atrolysin C at these two sites can be achieved at concentrations as low as 0.1 μM. Thus, concentrations of atrolysin C effective in cleaving aggrecan at both the Asn341-Phe342 and Glu373-Ala374 sites are within the range of concentrations of MMP-1 and MMP-3 (0.03–0.6 μM) reported to be present in synovial fluids of patients with arthritis (27, 28).

The pattern of BC-3-reactive fragments generated upon cleavage of aggrecan by atrolysin C was different from that which we previously found to be produced upon cleavage of cartilage aggrecan in response to interleukin-1 stimulation of bovine nasal cartilage (9). Although several bands of BC-3-reactive aggrecan fragments were seen in response to both endogenously generated aggrecanase and to atrolysin C, the high molecular mass band at 230 kDa, which represents the COOH-terminal aggrecan fragment formed upon initial cleavage by aggrecanase within the interglobular domain, was not generated by cleavage with atrolysin C. These data suggest that although atrolysin C has the ability to cleave at the Glu373-Ala374 bond, it does not exhibit the same preference as the cartilage aggrecanase for cleavage sites within the aggrecan molecule.

Although atrolysin C is a snake venom metalloproteinase and not found in many species, ADAM family members, which have been demonstrated in mammalian cell types, are related both in amino acid sequence and in domain structure to the reprolysin family of enzymes. ADAM-10,-12, and -15, which are both in amino acid sequence and in domain structure to the proline, are related and not found in many species, ADAM family members, which have been demonstrated in mammalian cell types, are related. ADAM family members, which have been demonstrated in mammalian cell types, are related.