

The Interglobular Domain of Cartilage Aggrecan Is Cleaved by PUMP, Gelatinases, and Cathepsin B*

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The action of three matrix metalloproteinases (MMPs), 72- and 95-kDa gelatinases (MMP-2 and MMP-9) and PUMP (MMP-7), and a cysteine proteinase, cathepsin B, were investigated on aggrecan the major proteoglycan of cartilage. All the enzymes cleaved aggrecan although the activity of the 95-kDa gelatinase was very low. Specific cleavage sites were investigated following incubation with a purified aggrecan G1-G2 domain fragment (150 kDa). Both gelatinases produced 110-kDa G2 and 56-kDa G1 products by a single cleavage at an Asn-Phe bond within the interglobular domain close to the G1 domain. This was similar to the action of stromelysin (MMP-3) (Fosang, A. J., Neame, P. J., Hardingham, T. E., Murphy, G., and Hamilton, J. A. (1991) *J. Biol. Chem.* **266**, 15579-15582). Cathepsin B also produced two fragments from a single cleavage at a Gly-Val bond only three amino acids C-terminal to the metalloproteinase cleavage site. PUMP cleaved at the metalloproteinase Asn-Phe site, but in addition produced a low yield of a smaller G2 fragment (56 kDa) corresponding to cleavage between Asp⁴⁴¹ and Leu⁴⁴² (human sequence), within the interglobular domain, close to the G2 domain. The apparent difference in size between the two G2 fragments released by PUMP (110 and 56 kDa) was much greater than predicted from the peptide length between the cleavage sites (100 amino acids). However, keratanase digestion greatly reduced the size of the 110-kDa G2 fragment, while producing only a small reduction in size of the 56-kDa product, showing that there was approximately 30–40 kDa of keratan sulfate attached to the interglobular domain between the PUMP cleavage sites. This new structural information on aggrecan may account for the previously observed stiffness of the interglobular domains when viewed by rotary shadowing electron microscopy (Paulsson, M., Morgelin, M., Wiedemann, H., Beardmore-Gray, M., Dunham, D. G., Hardingham, T. E., Heinegard, D., Timpl, R., and Engel, J. (1987)

Biochem. J. **245**, 763–772). These results show that in spite of a high keratan sulfate content the interglobular domain provides important sites for cleavage by different proteinases, including several members of the matrix metalloproteinase family.

Aggrecan is the major proteoglycan in cartilage. It is present at high concentration and is responsible for the compressive resilience of the tissue. One of the mechanisms for holding aggrecan within the cartilage matrix is the formation of high molecular weight aggregates in which up to a hundred or more proteoglycan monomers are bound by their G1 domains to a hyaluronan chain. Investigations of aggrecan turnover have shown that large fragments are slowly released from the tissues (1), which have lost their ability to bind to hyaluronan, suggesting a cleavage close to the G1 domain (2–5). We showed previously (6) that the metalloproteinase stromelysin makes a single cleavage between the aggrecan G1 and G2 domains at an Asn-Phe bond. Other studies have identified in interleukin-1-stimulated degradation, a different cleavage site at a Glu-Ala bond 30 amino acids C-terminal to the stromelysin site (7). The enzyme responsible for this cleavage, "aggrecanase," is as yet unidentified. It is significant that aggrecan fragments corresponding to aggrecanase action as well as the stromelysin action have been detected in extracts of human articular cartilage (8). Both are therefore active *in vivo*, but which enzymes have a major role in normal turnover and whether they differ from those most active in cartilage pathology remains to be determined.

Several classes of proteinases have been identified in cartilage including the metalloproteinases which are secreted enzymes active at acid and neutral pH. The expression of stromelysin and collagenase (both metalloproteinases) by chondrocytes and its modulation by inflammatory mediators such as IL-1¹ have been extensively studied (9–14). The 72- and 95-kDa gelatinases have also been identified in cartilage extracts, and the latter is induced by IL-1 and tumor necrosis factor-α in chondrocytes (15, 16). Cathepsin B, a cysteine proteinase, may also be active in cartilage. It has been identified in rheumatoid arthritic synovium (17) and in synovial fluids from rheumatoid arthritis and osteoarthritic patients (18). Although cysteine proteinases have pH optima at acid pH, they also have some activity close to neutral pH (17, 19).

Which proteinases are active in normal turnover of aggrecan and which are responsible for the enhanced rates of

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¹ The abbreviations used are: IL-1, interleukin-1, HPLC, high performance liquid chromatography.

degradation induced by IL-1 and tumor necrosis factor- α remains unknown. It is therefore important to investigate the actions of physiologically relevant enzymes such as the gelatinases and cathepsin B. We have also investigated PUMP, as this is closely related to the gelatinases and provides further information on what determines substrate specificity among the metalloproteinases.

EXPERIMENTAL PROCEDURES

Materials—Na¹²⁵I (IMS 30), Hyperfilm, and enhanced chemiluminescence reagents were from Amersham (Australia). Trypsin (diphenylcarbamoyl chloride treated) (EC 3.4.21.4), soybean trypsin inhibitor, 4-aminophenylmercuric acetate, 1,10-phenanthroline, 3-dimethylaminopropionitrile, 6-aminohexanoic acid, phenylmethanesulfonyl fluoride, benzamidine hydrochloride, 7-amino-4-methylcoumarin, and swine anti-rabbit-horseradish peroxidase were from Sigma. Agarose type HSC was from PS Park Scientific (Northampton, United Kingdom (U.K.)). A Bio-Sil SEC-400 HPLC column was from Bio-Rad (Sydney, Australia). Human umbilical chord hyaluronan was from BDH. Immobilon was from Millipore Waters (Sydney, Australia). Keratanase (*Pseudomonas* sp) was from ICN Biomedicals Australasia (New South Wales, Australia). Z-Phe-Arg-NHMec was from Bachem Feinchemikalien (Bubendorf, Switzerland). All other reagents were of analytical grade except guanidine HCl (Sigma) which was purified with activated charcoal and diatomaceous earth (Sigma).

Preparation of Cartilage Proteoglycan Aggregates, Proteoglycan Globular Domains, and Rabbit Antisera—Proteoglycan aggregates extracted from pig laryngeal cartilage in 4 M guanidinium hydrochloride, 50 mM sodium acetate buffer, pH 5.8, containing the proteinase inhibitors disodium EDTA, 6-aminohexanoic acid, benzamidine hydrochloride, and phenylmethanesulfonyl fluoride were purified on a CsCl density gradient (20). Proteoglycan aggregates from human (43-year-old male) were extracted in the same buffer and purified as described previously (21). Proteoglycan G1-G2 fragment was isolated from pig proteoglycan aggregates by mild trypsin digestion (22). The preparation of rabbit antisera against pig G1 and pig G2 core protein domains has been described elsewhere (22, 23).

Preparation and Activation of Enzymes—Recombinant human proPUMP cDNA (24) (a generous gift of Dr. Richard Breathnach) was expressed following transfection in a pEE12 vector into NSO (nonsecreting) mouse myeloma cells. Recombinant proPUMP was purified from culture medium by binding to S-Sepharose and elution with 200 mM sodium chloride followed by gel chromatography on a column of S-200 (25). Human 95- and 72-kDa progelatinases were prepared from culture media conditioned by U937 cells and human gingival fibroblasts, respectively (26). ProPUMP and progelatinases were activated by incubation for 1 h at 37 °C with 2 mM 4-aminophenylmercuric acetate, and the resulting activities were as described previously (25). Recombinant rat cathepsin B, expressed in *Saccharomyces cerevisiae* and purified by chromatography on DEAE-cellulose and Sephadex G-75 (27) was a generous gift of Dr. John Mort, Montreal, Canada. The activity of the cathepsin B, assayed with Z-Phe-Arg-NHMec as substrate at 30 °C and pH 5.5 (28) was 7.58 milliunits/mg.

Enzyme Digestions—Metalloproteinase digestions were carried out in buffer containing 10 mM calcium chloride, 100 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, at 37 °C. The digests were stopped by the addition of EDTA and 1,10-phenanthroline (10 and 2 mM final concentration, respectively). Cathepsin B digests were done in buffer containing 0.2 M sodium chloride, 1 mM EDTA, 10 mM dithiothreitol, 0.25 M sodium acetate, pH 5.5, at 37 °C. The digests were stopped by boiling for 5 min. Keratanase digestion of G1-G2 and G1-G2 fragments was done in 50 mM-Tris-acetate buffer, pH 7.5, at 37 °C for 18 h with 0.02 units of keratanase, 2 µg of substrate/60 µl.

Gel Electrophoresis and N-terminal Sequencing—G1-G2 digest products were analyzed on 5% sodium dodecyl sulfate-polyacrylamide gels (29), and protein bands were visualized by silver staining (30). For immunolocalization experiments, proteins were transferred onto Immobilon membranes, and G1 and G2 epitopes were identified using specific rabbit antisera, a swine-anti-rabbit antibody conjugated to horseradish peroxidase, followed by enhanced chemiluminescence using luminol substrate. Composite gels containing 1.2% polyacrylamide and 0.6% agarose were electrophoresed in a dissociative buffer containing 4 M urea (31). After electrophoresis proteoglycans were visualized by Toluidine Blue staining. Iodination of G1-G2 fragments

and localization of digest products on gels were as described previously (22, 32). N-terminal sequencing of isolated G2 products was as described by Sandy *et al.* (33).

RESULTS

Degradation of Cartilage Aggrecan—The ability of the proteinases 72- and 95-kDa gelatinase, PUMP, and cathepsin B to digest intact porcine aggrecan over 24 h was analyzed by composite gel electrophoresis (Fig. 1). Both PUMP and cathepsin B degraded the proteoglycan extensively producing several bands of faster migration. The 72-kDa gelatinase produced incomplete digestion as some undegraded aggrecan remained, however, the 95-kDa gelatinase under the conditions used showed little apparent activity. This was consistent with its reported low activity against proteoglycan (25). Parallel digests with human aggrecan showed similar comparative activity of the enzymes (results not shown), and the 95-kDa gelatinase again showed little apparent digestion.

Cleavage of Aggrecan G1-G2 Fragment—The intact G1-G2 domain fragment of porcine aggrecan was prepared and iodinated as described previously (6). This preparation enabled the pattern of cleavage of the different proteinases close to the G1 domain to be compared (Fig. 2). The 72- and 95-kDa gelatinases both produced two fragments which by immuno-

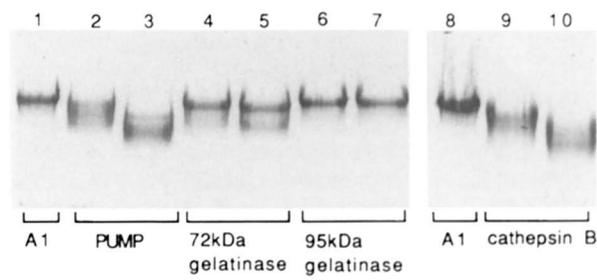


FIG. 1. Degradation of aggrecan by PUMP, 72- and 95-kDa gelatinase, and cathepsin B. Purified pig laryngeal aggrecan was analyzed before (lanes 1 and 8) and after digestion with 0.14 or 1.4 µg/ml PUMP (lanes 2 and 3, respectively), 2.3 or 23 µg/ml 72-kDa gelatinase (lanes 4 and 5, respectively), 4.1 or 41 µg/ml 95-kDa gelatinase (lanes 6 and 7, respectively), and 20 or 200 µg/ml cathepsin B (lanes 9 and 10, respectively). Samples containing 0.71 mg/ml (lanes 1–7) or 1 mg/ml (lanes 8–10) aggrecan were digested for 24 h at 37 °C and analyzed by dissociative agarose-acrylamide composite gel electrophoresis.

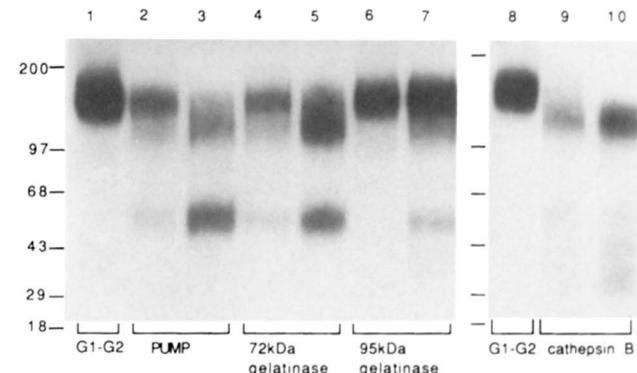


FIG. 2. Degradation of G1-G2 by PUMP, 72- and 95-kDa gelatinase, and cathepsin B. Purified ¹²⁵I-labeled G1-G2 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography before (lanes 1 and 8) or after digestion with 1.7 or 17 µg/ml PUMP (lanes 2 and 3, respectively), 0.8 or 8.0 µg/ml 72-kDa gelatinase (lanes 4 and 5, respectively), 5.8 or 58 µg/ml 95-kDa gelatinase (lanes 6 and 7, respectively), and 40 or 200 µg/ml cathepsin B (lanes 9 and 10, respectively). Samples were digested for 24 h at 37 °C.

blotting (22) were identified as a 110-kDa G2 fragment and a 56-kDa G1 fragment. PUMP also produced two major bands. The 110-kDa band was similar to that produced by the gelatinase, but the 56-kDa band was broader. Immunolocalization experiments showed both G1 and G2 domain fragments were present in the 56-kDa band, whereas the 110-kDa band contained G2 only, as with the gelatinases (Fig. 3). These results were compatible with PUMP cleaving at two sites, one similar to the gelatinase cleavage site, and the second cleaving some of the 110-kDa G2 fragment to release a 56-kDa G2 fragment.

Cathepsin B produced several cleavage products from the G1-G2 preparation (Fig. 2, lanes 9 and 10). A major 110-kDa band was again identified as containing G2 and no G1 domain. There were several diffuse bands of 56 kDa and less, which probably represented degraded G1 domains, although they were only poorly detected with anti-G1 antibodies. The reducing conditions used to promote cathepsin B activity are likely to have reduced some of the disulfide bonds in the G1 domain and this may have made more sites available for cathepsin B attack. Under reducing conditions we have previously found that stromelysin cleaves the G1 domain at sites that are not susceptible under non-reducing conditions.

Isolation and N-terminal Sequencing of Gelatinase and Cathepsin B-derived G2 Fragments—G2 fragments derived from gelatinase and cathepsin B digests were isolated by size exclusion chromatography on HPLC after mixing with hyaluronan to bind all G1 fragments and elute them in the void of the column (6, 22). For each digest, only one included peak corresponding to a G2 fragment was detected. Sequence analysis gave unambiguous results for both gelatinase digests. The G2 fragment had an N-terminal sequence Phe-Phe-Gly-Val. This showed that both gelatinases cleaved at the Asn-Phe site cleaved by stromelysins and shows this to be a site favored by several metalloproteinases. The G2 fragment produced by cathepsin B digestions had a different N-terminal sequence Val-Gly-Gly-Glu. This corresponds to a sequence three amino acids C-terminal to the gelatinase cleavage site. The proximity to the metalloproteinase site explains the similarity in size of this major G2 product of cathepsin B.

Isolation and N-terminal Sequencing of PUMP Digest Prod-

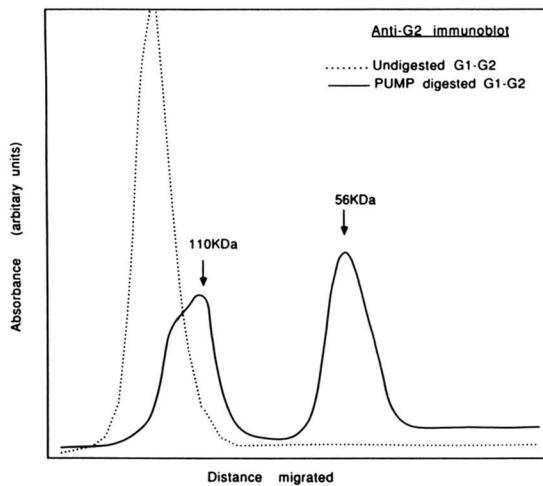


FIG. 3. Immunolocalization of G2 products following digestion of G1-G2 with PUMP. Purified G1-G2 (1.4 μ g) was digested with 0.2 μ g of PUMP in a total volume of 30 μ l for 24 h at 37 °C. Undigested (dotted line) and digested (solid line) G1-G2 were electrophoresed on a 5% gel and transferred to immobilon membranes for immunodetection with specific anti-G2 antisera. Bands were visualized on x-ray film by enhanced chemiluminescence using luminol, and the film was scanned on a laser densitometer (Pharmacia, Australia).

ucts—After mixing with hyaluronan and running on HPLC as described above, two included peaks of PUMP digests were identified (Fig. 4). Isolation and N-terminal sequencing of the larger 110-kDa fragment showed the same sequence as the 110-kDa G2 fragment produced by the gelatinases. The smaller 56-kDa peak also contained G2 but did not usually represent more than 20–30% of the total G2 detected. Sequence analysis showed two separate products to be present in the 56-kDa peak. One had an N-terminal sequence identical to the 110-kDa fragment (Phe-Phe-Gly-Val) whereas the other had an N-terminal sequence corresponding to a cleavage between Asp-Leu, where the aspartate represents residue 441 and 444 in the human and rat core protein sequences, respectively (34, 35) (Fig. 5). The latter sequence corresponded to a site in the interglobular domain close to G2. These results showed that PUMP cleaved with greatest activity at the main metalloproteinase site near G1, but also cleaved less frequently at a second site in the interglobular domain much closer to G2.

The second PUMP cleavage site cleaved the 110-kDa G2 product into two fragments of equal size (~56 kDa). This was surprising as the sequence of the interglobular domain between the two PUMP cleavage sites is only about 100 amino acids (~10 kDa). The explanation for this was provided by keratanase digestions, which reduced the apparent size of the 110-kDa G2 fragment to 70 kDa, but only decreased the size of the 56-kDa G2 fragment by ~10 kDa. A large proportion of the total keratan sulfate (~30–40 kDa) was therefore attached to the interglobular domain between the two PUMP cleavage sites (Fig. 6).

DISCUSSION

Our present results provide further evidence that the interglobular domain is a key site for the attack of aggrecan by proteinases. Cleavage in this region is important as it releases a major proteoglycan fragment from the G1 domain which binds it to aggregates. The results show that the gelatinases (MMP-2 and -9) and PUMP (MMP-7) cleave at the Asn-Phe site close to the G1 domain. We previously showed that stromelysins cleaved at this same site (6). Cathepsin B was also found to cleave very close to this site at a Gly-Val bond. Our results show that these metalloproteinases and cathepsin

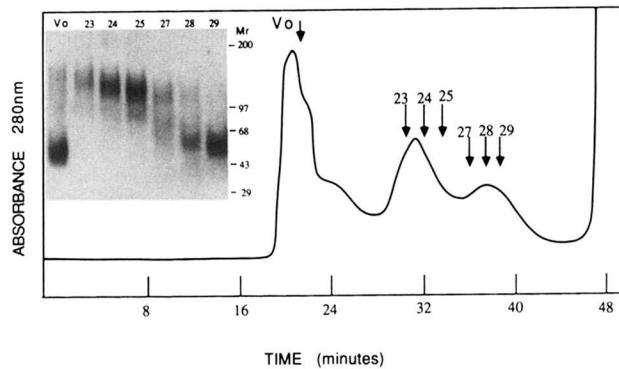


FIG. 4. Isolation of G2 and interglobular domain fragments produced by PUMP digestion of G1-G2. Purified G1-G2 (200 μ g) was digested with (0.4 μ g) PUMP in a total volume of 40 μ l for 24 h at 37 °C. The digestion products were mixed with 50 μ g of hyaluronan overnight at 4 °C, and the products were separated by size exclusion chromatography on a BioSil SEC 400 HPLC column. The column was eluted in buffer containing 0.1 M sodium sulfate, 0.123 M sodium chloride, 0.1 M sodium dihydrogen orthophosphate, pH 6.8, at 0.3 ml/min, and 0.4-ml fractions were collected. The inset shows aliquots of column fractions analyzed on 5% sodium dodecyl sulfate gels and visualized by silver stain.

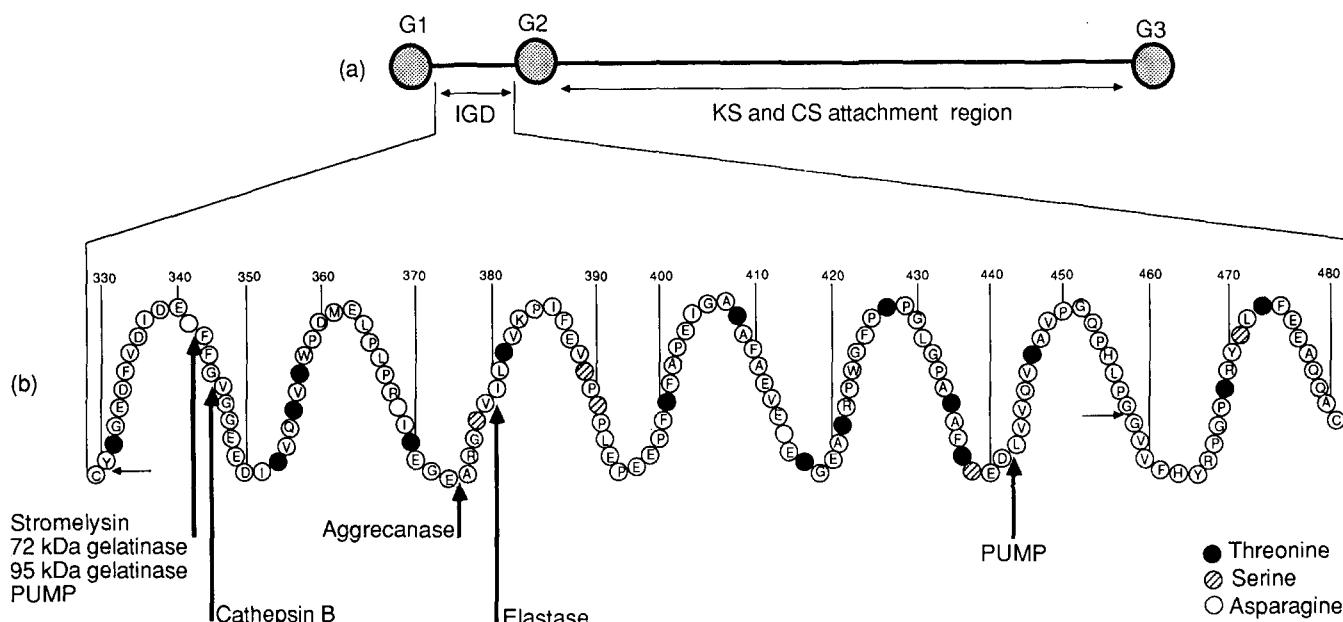
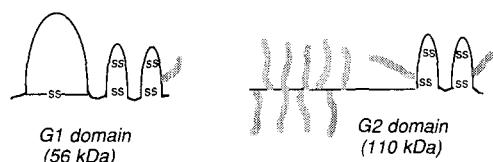


FIG. 5. Cleavage sites within the interglobular domain of aggrecan. *a*, schematic representation of aggrecan core protein showing the G1, G2, and G3 globular domains, the interglobular domain (IGD) between G1 and G2, and the extended keratan sulfate (KS) and chondroitin sulfate (CS) attachment region between G2 and G3. *b*, schematic representation of the amino acid sequence in human aggrecan IGD showing the cleavage sites identified for cathepsin B, gelatinases, PUMP, stromelysin (6, 8), leucocyte elastase (43), and aggrecanase, a cleavage site identified following IL-1 stimulation of articular cartilage (7). Filled, hatched, and blank circles designate potential glycosylation sites and represent threonine, serine, and asparagine residues, respectively. The horizontal arrows mark the interglobular domain boundaries (35, 44).

(a) G1 and G2 fragments produced by stromelysin, gelatinases and PUMP



(b) Minor G2 and IGD fragments produced by PUMP

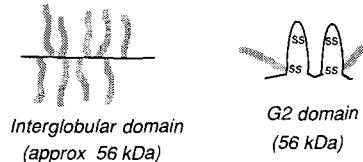


FIG. 6. Fragments produced by metalloproteinase digestion of G1-G2. Panel *a*, stromelysin, gelatinases, and PUMP cleave aggrecan G1-G2, yielding a 56-kDa G1 fragment and a 110-kDa G2 fragment. After keratanase treatment, the size of the G2 fragment was reduced to ~70 kDa. Panel *b*, PUMP made an additional cleavage in a small proportion (~20%) of 110-kDa G2 fragments, yielding a G2 fragment with mass 56 kDa, and an interglobular domain fragment of similar electrophoretic mobility. After keratanase treatment, the mass of the small G2 was reduced to only ~46 kDa. Thus, the bulk of the keratan sulfate chains present on the major 110-kDa G2 product are located on the 100 amino acid peptide derived from the interglobular domain.

B are not responsible for the aggrecanase cleavage site identified in IL-1-stimulated cartilage degradation (7) (Fig. 5). This implies that there is another unidentified enzyme induced by IL-1, which must be particularly active in cleaving the interglobular domain of aggrecan. However, there may still be participation of metalloproteinases in aggrecan turnover, as some G1 fragments in human cartilage have been shown to contain the C terminus of the metalloproteinase site

(8). Which of the metalloproteinases is responsible for cleavage at the Asn-Phe site *in vivo* is difficult to predict. Several of the metalloproteinases with the exception of PUMP are produced by chondrocytes. In culture the 72-kDa gelatinase is constitutively expressed, whereas the 95-kDa gelatinase (15, 16, 36), stromelysin (11-14), and collagenase (9, 10) are induced by cytokines. However, chondrocytes appear to behave differently in cartilage where separate expression of individual metalloproteinases has been observed, such as in the developing growth plate (37). Evidence in human articular cartilage suggested stromelysin to be most abundantly expressed and to increase with age, whereas gelatinase and collagenase were less abundant (38, 39). There are many factors that control metalloproteinase activity *in vivo* and the extent to which they are activated and how rapidly they become inhibited are difficult to determine experimentally, but are of key importance. The generation of plasmin by urokinase-type plasminogen activator is thought to be the key to stromelysin, collagenase, and 95-kDa gelatinase activation, while 72-kDa gelatinase may be regulated by a membrane activator (40).² A more complete analysis of all these parameters is therefore necessary before it can be determined which metalloproteinases are most active in normal and pathological situations in cartilage and how their activities complement those of other proteinases.

Very little is known about what determines substrate specificity among the metalloproteinases, and it is interesting that four different metalloproteinases with different substrate specificities all cleaved at the same Asn-Phe site in the interglobular domain. This included PUMP which differs from the others in lacking the large C-terminal hemopexin domain. In fibroblast collagenase the hemopexin domain has been shown to be important for binding to native triple helical collagen, and collagenase activity, but not caseinase activity, was lost on its removal (41). The results with PUMP show

² S. Atkinson and G. Murphy unpublished results.

that the hemopexin domain is not essential for determining activity at the Asn-Phe bond in the interglobular domain. PUMP also showed activity at a site not shared with the other metalloproteinases. This may reflect a less restricted substrate specificity of this metalloproteinase, or possibly greater access of the smaller enzyme lacking the hemopexin domain to a site inaccessible to the larger metalloproteinases.

The interglobular domain appears as an extended and stiffened segment 30 ± 5 nm long by rotary shadowing electron microscopy (42). This is shorter than the calculated fully extended sequence (~ 45 nm) and secondary structure prediction suggests it to be predominantly β sheet or random coil.³ Glycosylation is an important factor that may influence the protein configuration of the interglobular domain, its apparent stiffness, and the susceptibility to proteinases. There are 14 threonine, 4 serine, and 2 asparagine residues in the human sequence that may be glycosylated. Our analysis of the second PUMP cleavage site provides new evidence that up to 40 kDa of keratan sulfate is attached to the interglobular domain between the two PUMP sites (Fig. 6). This localizes most of the keratan sulfate attached to the G1-G2 region of aggrecan to a short 100 amino acid sequence separating the globular G1 and G2 domains. The keratan sulfate is likely to be in five to eight chains each 10–15 nm long, and they may make a major contribution to the apparent stiffness of this segment of the protein core. There may also be other O-linked or N-linked oligosaccharides. The precise distribution of these carbohydrate substitutions is not yet known, but amino acid sequencing of peptides from the interglobular domain has shown blank cycles at the position of several threonine and serine residues which suggests that there is carbohydrate attached to them.⁴

The sites of proteolytic cleavage reported within the interglobular domain appear to be grouped in short sequences that are free of sites for carbohydrate attachment and also of lower proline content (Fig. 5). However, variable glycosylation may influence the access of enzymes to some sites and the rather low efficiency of cleavage at the second PUMP site may be caused by glycosylation on some molecules at the Thr-Ser dipeptide (human residues 338–339) close to this cleavage site. The glycosylation of the interglobular domain may therefore under some circumstances influence the proteoglycans susceptibility to the proteolytic enzymes involved in its turnover.

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³ S. J. Perkins, personal communication.

⁴ P. J. Neame, unpublished results.

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