Metalloproteinase digestion of cartilage proteoglycan

Pattern of cleavage by stromelysin and susceptibility to collagenase

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The action of purified rabbit bone stromelysin was investigated on proteoglycan aggregates from pig laryngeal cartilage. The enzyme caused a rapid fall in viscosity of proteoglycan aggregate solution (6 mg/ml), and the products of a partial digest (60 % loss of relative viscosity) and a complete digest (95 % loss of relative viscosity) were characterized. Analysis by gel chromatography on Sepharose 2B under associative conditions showed that 95 % of the glycosaminoglycans in the complete digest were in small-sized fragments, whereas most of the hyaluronan-binding G1 domain and link protein remained intact and bound to hyaluronan. In contrast, there was extensive digestion of the G2 domain which resulted in 76 % loss in its detection by immunoassay. Analysis of the partial digest also showed considerable loss (40 %) of detection of the G2 domain, but the glycosaminoglycan-rich fragments were much larger than in the complete digest. There was also much less cleavage to create small fragments containing the G1 domain. This was evident on SDS/PAGE analysis where a 58 kDa G1 domain fragment was abundant in the complete digest, but was only present in small amounts in the partial digest. There was also only very limited conversion of link protein from a 44 kDa form to a 40 kDa form. The digestion of proteoglycan aggregate (6 mg/ml) by stromelysin was unaffected by the addition of a high concentration of extra chondroitin sulphate chains (14 mg/ml), and the digestion of proteoglycan monomer showed that the G1 domain was resistant to stromelysin digestion even when not bound to hyaluronan and link protein. The results show that stromelysin degrades the proteoglycan protein core with major cleavages close to, but not within, the G1 domain, and extensive cleavage in other regions. Experiments with purified collagenase, a metalloproteinase structurally related to stromelysin, showed that it too cleaved proteoglycan at several sites within the glycosaminoglycan-rich region of the core protein. Metalloproteinase attack on proteoglycan thus not only occurs with stromelysin but also with collagenase.

INTRODUCTION

Proteoglycans and collagen are the major structural components of the extracellular matrix of cartilage. Collagen provides the tensile strength and the proteoglycans are largely responsible for the compressive stiffness of the tissue (Kempson *et al.*, 1970, 1976). The major proteoglycan present in cartilage is the high-molecular-mass aggregating species. A proteoglycan aggregate is composed of a central filament of hyaluronan to which numerous proteoglycan monomers are attached (Hardingham & Muir, 1972; Heinegård & Hascall, 1974; Hardingham *et al.*, 1976). The interaction of the proteoglycan monomer with hyaluronan is further stabilized by a link protein (Hardingham, 1979). The structure of the proteoglycan monomer (see Fig. 1) consists of a protein core containing three globular domains (Wiedemann *et al.*, 1984; Paulsson *et al.*, 1987; Mörgelin *et al.*, 1988): G1, G2 and G3. The G1 domain (*N*-terminal) interacts in a highly specific way with hyaluronan, but the functions of the G2 and G3 domains are unknown (Fosang & Hardingham, 1989). There is a short extended region that joins G1 and G2 and a long extended domain that forms the major glycosaminoglycan attachment region between G2 and G3 (Doege *et al.*, 1986). The chondroitin sulphate chains are restricted to the long extended region between G2 and G3, whereas the keratan sulphate chains are distributed more widely throughout the core protein. Many keratan sulphate chains are concentrated in a keratan-sulphate-rich region close to the G2 domain and adjacent to the chondroitin sulphate domain. Some *O*-linked and *N*-linked oligosaccharides are also present on the protein core.

The turnover of cartilage proteoglycans involves a regulated mechanism of synthesis and secretion by the chondrocytes and degradation in the cartilage matrix. This involves the digestion of monomer within the aggregate structures. *In vitro* studies of normal and interleukin 1 (IL-1)-stimulated turnover have shown that there is proteolytic cleavage of the core protein to release a large glycosaminoglycan-bearing fragment which has lost its ability to aggregate with hyaluronan (Ratcliffe *et al.*, 1986). The agents and mechanisms actually responsible for the physiological turnover have not been fully elucidated. The neutral metalloproteinases (collagenase, gelatinase and stromelysin), which have been isolated in small amounts from cartilage (Sapolsky *et al.*, 1976; Martel-Pelletier *et al.*, 1988) and are synthesized and secreted by chondrocytes (Murphy *et al.*, 1986, 1989), may be involved in these processes. The secretion of stromelysin and collagenase is increased in systems undergoing enhanced turnover, for example during IL-1 treatment, when stromelysin gene expression is also induced (Frisch & Ruley, 1987).

The purpose of this work was to study the action of stromelysin on the large aggregating proteoglycan. In the present study we have characterized the cleavage products obtained after incubation of purified pig laryngeal proteoglycan aggregates with purified stromelysin from rabbit bone culture medium (Galloway *et al.*, 1983). Conditions were chosen to give a relatively high concentration of proteoglycan and low amounts of enzyme, so that results would be more relevant to proteoglycan degradation within the cartilage matrix.

Abbreviations used: IL-1, interleukin 1; TIMP, tissue inhibitor of metalloproteinase.

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Fig. 1. Schematic representation of proteoglycan aggregate and its digestion by stromelysin

The major protein and carbohydrate components of proteoglycan are shown together with the interaction with link protein and hyaluronate involved in aggregation. The general patterns of cleavage in partial and complete digests are shown. These indicate the approximate number of major cleavage sites and show that the G1 domain and link protein survive digestion with stromelysin, whereas a major fraction of G2 is destroyed. The fate of the G3 domain was not investigated.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade (BDH Chemicals, Poole, Dorset, U.K.), except for guanidine hydrochloride, which was purified with activated charcoal (Norit GSX; Hopkin & Williams, Chadwell Heath, Essex, U.K.). 6-Aminohexanoic acid, benzamidine hydrochloride, phenylmethanesulphonyl fluoride, 3,3'-diaminobenzidine tetrahydrochloride and anti-(rabbit IgG) antibody-peroxidase conjugate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Sepharose gels were obtained from Pharmacia (Uppsala, Sweden). Na^{125}I was obtained from Amersham International (Amersham, Bucks., U.K.).

Preparation of proteoglycan aggregate (A1) and monomer (A1D1)

Pig laryngeal cartilage was obtained fresh from the slaughterhouse. It was dissected free of adhering tissue and perichondrium and then finely shaved with a Stanley Surform, and the proteoglycan was extracted with 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 6.8, for 24 h at 4 °C with continuous mixing. The extraction buffer contained the following proteinase inhibitors: EDTA (10 mM), 6-amino-hexanoic acid (100 mM), benzamidine hydrochloride (5 mM) and phenylmethanesulphonyl fluoride (0.1 mM). Extracts were filtered through glass wool and the filtrates were dialysed against 7 vol. of water containing 50 mM-sodium acetate and the same concentrations of inhibitors to allow reassociation of the proteoglycan aggregates. Dialysed cartilage extracts were adjusted to a density of 1.6 g/ml with CsCl and centrifuged at 100 000 g for 18 h at 10 °C. The aggregated proteoglycan (A1) was isolated from the bottom 8 ml of the gradient (total volume 34 ml) and was dialysed against the enzyme buffer (0.05 M-Tris/HCl, pH 7.5, containing 10 mM- CaCl_2 and 100 mM-NaCl). The final concentration of the proteoglycan was determined from the content of hexuronate (25%, w/w). Proteoglycan monomer (A1D1) was prepared from the proteoglycan aggregate

by fractionation in a dissociative CsCl density gradient (Hardingham, 1979). The monomer was dialysed against the enzyme buffer and its final concentration was determined from the content of hexuronate (25%, w/w).

Preparation of metalloproteinases

Rabbit bone stromelysin was purified from 4-amino-phenylmercuric-acetate-activated rabbit bone culture medium as described by Galloway *et al.* (1983). The specific activity of the enzyme (24 kDa) was 2400 units/mg in the [^{14}C]casein assay, where 1 unit degrades 1 μg of casein/min to peptides soluble in 3% (w/v) trichloroacetic acid.

Human gingival fibroblast procollagenase was prepared as described by Whitham *et al.* (1986) and its recombinant form from mouse C127 fibrosarcoma cells was prepared as described by Cooksley *et al.* (1990). To ensure that the former contained no stromelysin, it was further purified by passage through a column of sheep anti-(human stromelysin) IgG linked to Sepharose (Okada *et al.*, 1986). Both forms of the enzyme migrated as a typical doublet on SDS/PAGE and were assumed to have an A_{280} of 10. Procollagenase preparations were activated by incubation with 2 mM-4-aminophenylmercuric acetate at 37 °C for 4 h (Murphy *et al.*, 1987).

General analytical procedures

Hexuronate was determined by an automated procedure (Heinegård, 1973) of the modified carbazole reaction (Bitter & Muir, 1962), with glucuronolactone as standard. Sulphated glycosaminoglycan concentration was determined by using an automated modification (Ratcliffe *et al.*, 1986) of the method of Farndale *et al.* (1982) with whale and shark chondroitin sulphate as a standard. Radioimmunoassays for G1 domain, link protein (Ratcliffe & Hardingham, 1983), G2 domain (Fosang & Hardingham, 1989) and keratan sulphate (Zanetti *et al.*, 1985) were essentially as described by Caterson *et al.* (1979).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out on slab gels with a 5% (w/v) polyacrylamide concentration, essentially as described by Fairbanks *et al.* (1971). Protein bands were detected by silver staining (Morrissey, 1981). Molecular mass calibrations were made with globular protein standards (12.3–200 kDa) obtained from BRL. Immunoblotting with anti-G1 and anti-G2 antisera (Fosang & Hardingham, 1989) was performed by a modification of the method of DeBlas & Cherwinski (1983). Composite agarose/polyacrylamide-gel electrophoresis was carried out on slab gels using a modification (Carney *et al.*, 1986) of the method of McDevitt & Muir (1971). Gels were run under dissociative conditions in 4 M-urea and proteoglycan bands were detected by staining with Toluidine Blue.

Gel chromatography

Digests of proteoglycan aggregates were fractionated on a column (130 cm $\times$ 9 mm) of Sepharose 2B or CL-2B eluted with 0.5 M-sodium acetate, pH 6.8, at 4 °C or with 2 M-guanidine hydrochloride/50 mM-sodium acetate, pH 5.8, at 4 °C respectively. Fractions were analysed for total sulphated glycosaminoglycans and by radioimmunoassay for G1 domain, G2 domain, link protein and keratan sulphate.

Digestion of proteoglycan aggregates with stromelysin

Proteoglycan aggregate in enzyme buffer was mixed with stromelysin such that the final concentration of substrate was 6 mg/ml and the final concentration of enzyme was 2.0 units/ml of substrate. The digest mixture was incubated in a viscometer bath at 37 °C and the progress of the digestion was monitored by

measuring the decrease in relative viscosity determined at regular intervals. After an incubation period of 24 h the relative viscosity had reached a limiting value. The digest was terminated with EDTA (10 mM) and 1,10-phenanthroline (2 mM) which inhibit the enzyme.

To obtain a partial digest, proteoglycan was mixed with stromelysin (0.2 units/ml of substrate). The digest mixture was incubated in a viscometer bath at 37 °C and was terminated as previously described when a decrease of 65% of the initial viscosity had been achieved.

RESULTS

Partial and complete digests of proteoglycan aggregate with stromelysin

The ability of the purified rabbit bone culture stromelysin to degrade cartilage proteoglycan was tested on the pig laryngeal cartilage aggregate preparation (6 mg/ml). With a high concentration of enzyme (2 units/ml) there was a rapid fall in relative viscosity, with a loss of 90% in 3 h and reaching a limit of a 95% decrease by 24 h. A partial digest with one-tenth of the enzyme concentration (0.2 unit/ml) gave a 65% loss of relative viscosity in 6 h, and the fragments produced under these conditions were also investigated further. The characteristics of the digest products were assessed initially by composite agarose/polyacrylamide-gel electrophoresis (McDevitt & Muir, 1971; Carney *et al.*, 1986) (Fig. 2). The control proteoglycan aggregate preparation when dissociated in urea shows a single main band stained with Toluidine Blue. After digestion, the stained glycosaminoglycan-rich fragments were of higher mobility. They formed a broad band in the partial digest, which was resolved into two quicker-migrating bands in the complete digest (Fig. 2, track 3).

Details of the size and composition of the digest products were investigated by gel chromatography under associative and dissociative conditions. The column fractions were analysed for link protein and a range of proteoglycan protein components (G1 and G2 domains) and glycosaminoglycan components (total glycosaminoglycans and keratan sulphate) (see Fig. 1). The results for the limit digest (Fig. 3c) showed that, under associative conditions, a high proportion of proteoglycan-binding region (G1 domain) remained bound to hyaluronate together with link

Fig. 2. Electrophoresis on a composite agarose/polyacrylamide gel of proteoglycan after partial and complete digestion with stromelysin

Pig laryngeal proteoglycan aggregate (6.0 mg/ml) was incubated at 37 °C with stromelysin at 0.2 unit/ml for the 6 h partial digest (track 2) or 2.0 units/ml for the 24 h complete digest (track 3). Track 1 is the control incubation for 24 h without enzyme. The samples were dialysed, freeze-dried and dissolved in 8 M-urea, and samples corresponding to 5 µg of hexuronate were applied to each track of a composite agarose/polyacrylamide gel run in 4 M-urea and stained with Toluidine Blue (Carney *et al.*, 1986).

protein at the excluded volume of the column. In contrast, very little G2 domain remained attached to the G1-link protein-hyaluronate complex. Most of the total sulphated glycosaminoglycans (95%) were cleaved from the aggregate and were eluted as small fragments (K_{av} 0.3–0.8). Under dissociative conditions the G1 domain was eluted as a low-molecular-mass component close to link protein, confirming that it was cleaved from the major glycosaminoglycan-bearing fragments.

The presence of the G2 domain was not detected amongst the different released proteoglycan fragments of various sizes. Quantitative analyses of G2 in the control undigested proteoglycan and in the limit digest showed that there was a 76% loss of detection of G2 epitopes after digestion, whereas there was no loss of detection of the G1 domain or link protein. It therefore appeared that G2 was significantly degraded by stromelysin, whereas the G1 domain and link protein were resistant to digestion.

These results established that there were several stromelysin cleavage sites within proteoglycan that are accessible to digestion in the aggregate under non-denaturing conditions (Fig. 1). In order to investigate those sites most susceptible to cleavage, the digest products from the partial digest were analysed. Gel chromatography of the products (Fig. 4c) showed under associative conditions a large increase in the total sulphated glycosaminoglycans in retarded non-aggregated fragments (60%, compared with 15% in the undigested preparation). As in the

Fig. 3. Gel chromatography of control and limit stromelysin digests of proteoglycan aggregate

The control (a and b) and limit-digested (c and d) proteoglycan aggregates were chromatographed on a column of Sepharose 2B under associative conditions (a and c) or on Sepharose CL-2B under dissociative conditions (b and d), as described in the Materials and methods section. The eluted fractions were analysed for sulphated glycosaminoglycans by the dye-binding assay (●) and for G1 (---), link protein (LP, ····), keratan sulphate (KS, □) and G2 (Δ) by radioimmunoassays as described in the Materials and methods section.

Fig. 4. Gel chromatography of control and partial stromelysin digests of proteoglycan aggregate

The control (a and b) and partially digested (c and d) proteoglycan aggregates were chromatographed on a column of Sepharose 2B under associative conditions (a and c) or on Sepharose CL-2B under dissociative conditions (b and d) as described in the Materials and methods section. The fractions were analysed for sulphated glycosaminoglycans by the dye-binding assay (●) and for G1 (---), link protein (LP, ····), keratan sulphate (KS, □) and G2 (△) by radioimmunoassays as described.

limit digest, most of the link protein and G1 domain remained at the void volume bound to hyaluronate, but in contrast to the limit digest these fractions also contained much of the keratan sulphate and the surviving G2 domain. Quantitative analysis also showed a partial loss of detection of the G2 domain, but the loss was less (40 %) than in the limit digest (76 %). The average size of the glycosaminoglycan-rich fragments released was much larger in the partial digest than in the total digest [K_{av} 0.15–0.6 (Fig. 4c) compared with 0.3–0.8 (Fig. 3c)]. Under dissociative conditions the average size of these products [K_{av} 0.1–0.6 (Fig. 4d)] was only slightly smaller than that of the undigested monomers [K_{av} 0.05–0.5 (Fig. 4b)] and some G1 domain was still attached, but there were also smaller-size fragments containing significant amounts of keratan sulphate and G1 domain. These were a considerably larger apparent size (K_{av} 0.5–0.7) than the peak of free G1 domain, which accounted for only about 10 % of the total G1 domain.

The effect of digestion on link protein and the size of the released G1 domain were investigated further by SDS/PAGE analysis (Fig. 5). The undigested proteoglycan contained only one link protein band, as pig laryngeal cartilage contains mainly the more glycosylated form (Hardingham, 1979), and this persisted in both the digests. A small amount of a faster-migrating link protein band was present in the partial digest, but it did not increase in abundance in the limit digest. Most of the link protein was therefore completely resistant to stromelysin

Fig. 5. SDS/PAGE analysis of proteoglycan aggregate before and after digestion with stromelysin

Proteoglycan aggregate (6 mg/ml) was incubated with stromelysin at 0.2 unit/ml for a 6 h partial digest (track 2) or 2.0 units/ml for a 24 h complete digest (track 3). The undigested control is in track 1. Samples (3 µg of protein) were loaded on SDS/PAGE (5 % gel). After electrophoresis the gel was silver-stained (Morrissey, 1981). The large proteoglycan fragments remain at the origin of the gel.

digestion. In the limit digest there was, in addition to the link protein bands, a major protein band (apparent molecular mass 58 kDa) which was identified by immunoblotting (result not shown) as the G1 domain. In the partial digest only a small amount of this fragment was present.

It was noticeable that in both partial and limit digests there was little evidence of small fragments containing the G2 domain. This was investigated further by immunolocalizing G2 after SDS/PAGE analysis of the digest products. The results showed that even in the limit digest there were no small (< 100 kDa) fragments reactive with the anti-G2 antiserum (results not shown). There were only two weak diffuse bands at about 100 kDa and 120 kDa, which appeared from the weak silver staining in this region (Fig. 5) to be of low abundance. The major loss of G2 therefore appeared to result from its cleavage by stromelysin and the inability of the fragments produced to compete with native G2 domain in the radioimmunoassay.

Effect of chondroitin sulphate on stromelysin digestion of proteoglycan

In investigating the action of stromelysin on proteoglycan at high substrate concentrations, it was possible that the action of the enzyme on the protein core may be influenced by the very high concentration of polyanionic glycosaminoglycans that make up 90 % of the proteoglycan composition. A digest was therefore carried out to compare the rate of digestion of proteoglycan at 6 mg/ml with and without the addition of chondroitin sulphate (14 mg/ml). Gel chromatography of the digest products showed an identical degree of release of glycosaminoglycan-rich fragments from the aggregate (Fig. 6). The increased concentration of glycosaminoglycan therefore appeared to have little effect on the rate at which stromelysin degraded the protein core substrate. Proteoglycan at 20 mg/ml was also digested by stromelysin and produced a similar pattern of fragmentation, although the rate of digestion was slower (results not shown).

Stromelysin digestion of proteoglycan monomer

The proteoglycan G1 domain appeared very resistant to digestion by stromelysin when in the aggregate structure. This might have been because it is tightly bound to hyaluronan and

Fig. 6. Gel chromatography of proteoglycan aggregate digested by stromelysin in the presence and the absence of free chondroitin sulphate chains

Proteoglycan aggregates (6 mg/ml) were incubated in the presence (●) or absence (○) of free chondroitin sulphate chains (14 mg/ml) with stromelysin (0.2 unit/ml) for 10 h at 37 °C. A sample of the digest was chromatographed on a column of Sepharose 2B under associative conditions as described in the Materials and methods section. The eluted fractions were analysed for sulphated glycosaminoglycans by the dye-binding assay.

associated with link protein. Its sensitivity to digestion was therefore investigated in a preparation of proteoglycan monomer with no hyaluronan or link protein present. After incubation for 24 h the digest products were mixed with hyaluronan and fractionated by gel chromatography in order to test how much G1 domain retained hyaluronate-binding properties (Fig. 7). Most (85%) of the sulphated glycosaminoglycans were in fragments (K_{av} 0.2–0.7) which were unable to bind to hyaluronate, whereas with control monomer (Fig. 7a) only 30% was not bound to hyaluronate. However, more than 90% of the G1 domain appeared to bind to hyaluronate. Its resistance to stromelysin digestion was thus independent of its interaction with hyaluronate and link protein.

Cartilage proteoglycan digestion by collagenase

Cartilage proteoglycan was incubated with human gingival fibroblast collagenase in order to test if the sensitivity of proteoglycan as a substrate for stromelysin was a basis for discriminating between the actions of stromelysin and collagenase. Viscometric analysis showed that there was a 33–39% fall in relative viscosity over 24 h, which was > 90% inhibited by tissue inhibitor of metalloproteinase (TIMP) or 10 mM-EDTA/2 mM-1,10-phenanthroline. Investigation of the products on composite agarose/polyacrylamide-gel electrophoresis (Fig. 8) confirmed that considerable digestion had occurred. With a low amount of enzyme there were increases in electrophoretic mobility and with more extensive digestion the band was resolved into two main fast-migrating components. The major band of these two was of slightly lower electrophoretic mobility than the main band produced by stromelysin digestion (Fig. 2). The digest products were similar whether the collagenase was a purified recombinant or a non-recombinant enzyme preparation.

The sensitivity of the digestions to inhibition by TIMP and EDTA/1,10-phenanthroline clearly confirmed the activity to be a metalloproteinase. As digestion was achieved with recombinant collagenase, expressed in *Escherichia coli*, as well as with collagenase purified from human gingival fibroblasts, it is unlikely

Fig. 7. Gel chromatography of control and limit digests of proteoglycan monomer

Proteoglycan monomer (6.0 mg/ml) before (a) and after (b) digestion with stromelysin (2.0 units/ml) was incubated with hyaluronate (2%, w/w) and was then chromatographed on a column of Sepharose 2B under associative conditions as described in the Materials and methods section. The eluted fractions were analysed for sulphated glycosaminoglycans (●) by the dye-binding assay and for G1 (---) and keratan sulphate (KS, □) by radioimmunoassay as described.

Fig. 8. Electrophoresis on a composite agarose/polyacrylamide gel of proteoglycan aggregate after digestion with human gingival fibroblast collagenase

Pig laryngeal proteoglycan aggregate (6.0 mg/ml) was incubated without (tracks 1 and 4) or with collagenase (track 2, 31 µg/ml; track 3, 3.1 µg/ml) for 24 h at 37 °C. Electrophoretic conditions and staining were as in Fig 2.

that activity was due to a contaminant metalloproteinase. Immunochemical tests for stromelysin in the fibroblast collagenase preparation were also negative (results not shown). The ability of purified collagenase to digest proteoglycan was observed in all preparations tested (four from fibroblast cell sources and two recombinant preparations). The pattern of

cleavage and the rate of digestion were also similar with proteoglycan aggregate or monomer as substrate (results not shown).

DISCUSSION

Stromelysin was able to degrade native aggregated proteoglycans at substrate concentrations up to at least 20 mg/ml *in vitro*. A limit digest of proteoglycan aggregate produced a number of cleavages along the protein core, including the release of the G1 domain from major glycosaminoglycan-bearing fragments (Fig. 1). The glycosaminoglycan-rich fragments were estimated to have average molecular masses of 400 kDa (Ohno *et al.*, 1986). As the proteoglycan molecular mass is about $(1.5\text{--}2.0) \times 10^3$ kDa (Hardingham, 1981), this suggests that three to five cleavage sites are present within the chondroitin-sulphate-rich region of the protein core (Fig. 1). Partial digests resulted in the release of much larger glycosaminoglycan fragments with fewer internal cleavages (Fig. 1). There was a substantial loss of G2 epitopes even in the partial digest. This suggests that there are favoured cleavage sites around the G2 domain which are amongst the first cleaved by stromelysin. This provides an efficient means for separating the region containing the G1 domain from the glycosaminoglycan-rich part of the molecule. The G1 domain was not degraded by stromelysin, either as part of an aggregate in a conformation where it was protected by hyaluronan and link protein or when it was exposed on monomer proteoglycan (Fig. 7). Conversely, the G2 domain appeared to be more vulnerable to degradation by stromelysin, as shown by the loss of G2 epitopes. Analysis of proteoglycan degradation in articular cartilage explants showed there was a significant loss of both G1 and G2 epitopes in IL-1-stimulated cultures, although free G1 and free G2 fragments, but no G1-G2 fragments, were also released into the medium (Fosang *et al.*, 1991a). In addition, it appears that the products obtained from the digestion of purified preparations of proteoglycans with stromelysin have some similarities to the breakdown products of proteoglycans extracted from cartilage (Mort *et al.*, 1983) and to those reported to be released in normal and IL-1-stimulated cell and explant cultures (Tyler, 1985a,b; Ratcliffe *et al.*, 1986). Recent work (Fosang *et al.*, 1991b) has identified a stromelysin cleavage close to the G1 domain at an Asn-Phe-Phe site, but this differs from a Gly-Glu-Ala-Arg cleavage site between the G1 and G2 domains detected as a product in IL-1-stimulated cartilage cultures (Sandy *et al.*, 1991). The results suggest, however, that although stromelysin may be involved in the initial attack on aggregating proteoglycans in normal and IL-1-stimulated turnover, there are other enzymes involved which digest the G1 domain and produce a range of smaller fragments from the rest of the protein core.

SDS/PAGE of the digest products demonstrated the presence of link protein of decreased molecular mass which was absent from the control digest. This corresponds to the cleavage of link protein into a fragment of similar size to the major trypsin-cleaved product (Bonnet *et al.*, 1985), but the extent of the conversion was quite small even at the later stages of digestion. The cleavage of link protein from human neonatal proteoglycan aggregate digested with a cartilage-derived stromelysin isolated from IL-1-stimulated human articular cartilage has been well characterized by Nguyen *et al.* (1989). The human link protein was cleaved at the His-16-Ile-17 bond and, given sufficient time, it was degraded to a single protein band that co-migrated with the smallest native link protein component. The present results suggest that in pig link protein only a few molecules are particularly sensitive to digestion, whereas most are rather resistant. This may result from differences in glycosylation, as cDNA cloning (Dudhia & Hardingham, 1989, 1990) has shown

the same amino acid sequences to be present in this region of human and pig link proteins. Other studies involving the characterization of degradation products of proteoglycans produced by undefined metalloproteinases from stimulated culture systems have also reported the accumulation of isolated G1 domain fragments comparable with those detected in the present study (Morales & Kuettner, 1982; Campbell *et al.*, 1986; Nguyen *et al.*, 1989; Pelletier *et al.*, 1988).

Much of the work involved in characterizing the metalloproteinases has defined the major natural substrates with which they are active. Collagenase is a unique mammalian enzyme in having an action that cleaves the triple helical collagen domain. This is not an activity shared with the other metalloproteinases. Stromelysins have frequently been referred to as 'proteoglycanases', although it is clear that many proteinases are able to cleave the proteoglycan protein core. The actions described here on proteoglycan do not suggest that there are sites of cleavage by stromelysin on the proteoglycan protein core in regions or domains which are inaccessible to other proteinases. The action of stromelysin therefore does not appear to be highly specialized for proteoglycan cleavage. Furthermore, this enzyme has also been shown to digest many other matrix protein substrates. The ability of collagenases to cleave proteoglycan shows that it is not essential for substrates of collagenase to be triple-helical and collagenous. The unique action of collagenase on collagen is thus not its only activity, as it can also degrade proteoglycans. However, it is possible that collagen is greatly preferred to proteoglycan as a substrate by collagenase under the physiological conditions in the tissue.

We thank Andy Docherty and colleagues (Celltech Ltd., Slough, U.K.) for crude C127 culture medium containing recombinant human collagenase. We are grateful to the Arthritis and Rheumatism Council for support.

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Received 23 April 1991/19 June 1991; accepted 24 June 1991