Fibroblast and neutrophil collagenases cleave at two sites in the cartilage aggrecan interglobular domain

Amanda J. FOSANG,*¶ Karena LAST,* Vera KNÄUPER,† Peter J. NEAME,‡ Gillian MURPHY,§ Timothy E. HARDINGHAM,‖ Harald TSCHESCHE† and John A. HAMILTON*

*University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Royal Parade, Parkville, Victoria 3050, Australia.
†Universitat Bielefeld, Fakultat für Chemie, Lehrstuhl für Biochemie, W-4800 Bielefeld, Federal Republic of Germany.
‡Shriners Hospital for Crippled Children, Tampa, FL 33612, U.S.A., §Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, U.K., and ‖Kennedy Institute of Rheumatology, 6 Bute Gardens, Hammersmith, London W6 7DW, U.K.

The actions of recombinant human fibroblast collagenase (MMP1), purified polymorphonuclear leucocyte collagenase (MMP8) and their N-terminal catalytic domain fragments against cartilage aggrecan and an aggrecan G1–G2 fragment have been investigated *in vitro*. After activation with recombinant human stromelysin and trypsin, both collagenases were able to degrade human and porcine aggrecans to a similar extent. An N-terminal G1–G2 fragment (150 kDa) was used to identify specific cleavage sites occurring within the proteinase-sensitive interglobular domain between G1 and G2. Two specific sites were found; one at an Asn^{341}-Phe^{342} bond and another at Asp^{441}-Leu^{442} (human sequence). This specificity of the collagenases for aggrecan G1–G2 was identical with that of the truncated metalloproteinase matrilysin (MMP7), but different from those of stromelysin (MMP3) and the gelatinases (MMP2 or gelatinase A; MMP9 or gelatinase B) which cleave at the Asn-Phe site, but not the Asp-Leu site. In addition, collagenase catalytic fragments lacking C-terminal hemopexin-like domains were tested and shown to exhibit the same specificities for the G1–G2 fragment as the full-length enzymes. Thus the specificity of the collagenases for cartilage aggrecan was not influenced by the presence or absence of the C-terminal domain. Together with our previous findings, the results show that stromelysin-1, matrilysin, gelatinases A and B and fibroblast and neutrophil collagenases cleave at a common, preferred site in the aggrecan interglobular domain, and additionally that both fibroblast and neutrophil collagenases cleave at a second site in the interglobular domain that is not available to stromelysin or gelatinases.

INTRODUCTION

The matrix metalloproteinases, which include the collagenases, gelatinases and stromelysins, are believed to be important mediators of cartilage destruction occurring in joint pathology. Elevated levels of collagenase and stromelysin have been observed in human and canine experimental OA cartilage (Pelletier et al., 1983; Martel-Pelletier et al., 1984; Pelletier and Martel-Pelletier, 1985; Dean et al., 1987; Okada et al., 1992), and the levels of stromelysin and collagenase found in synovial fluids of patients with rheumatoid arthritis and osteoarthritis are also increased (Walakovits et al., 1992; Lohmander et al., 1993). The genes for collagenase and stromelysin are induced *in vitro* in chondrocytes by cytokines such as interleukin-1 and tumour necrosis factor α (Murphy et al., 1986; Hasty et al., 1990b; Richards et al., 1991; Mitchell and Cheung, 1991). We have shown previously that stromelysin, gelatinases and matrilysin degrade the main cartilage proteoglycan, aggrecan, and we have identified specific cleavage sites within the proteinase-sensitive interglobular domain (Fosang et al., 1991, 1992). Purified fibroblast collagenase has been shown to degrade cartilage proteoglycan *in vitro* (Hughes et al., 1991); however, the identification of the specific cleavage sites recognized by this enzyme, or by the neutrophil collagenase, has not been described.

Two distinct mammalian collagenase gene products have been identified (Whitham et al., 1986; Wilhelm et al., 1986; Hasty et al., 1990a; Knäuper et al., 1990) and shown to share certain structural and biochemical properties. The domain structures of the fibroblast (MMP1) and neutrophil (MMP8) collagenases are very similar, incorporating pro-peptide domains, catalytic domains and a C-terminal domain that is thought to confer substrate specificity (Clark and Cawston, 1989; Murphy et al., 1992). Both collagenases uniquely cleave the three α chains of types I, II and III collagens at a single locus, producing characteristic TC^A and TC^B fragments at 25 °C. Different specificities for collagen substrates have been observed (Welgus et al., 1981b; Hasty et al., 1987; Mallya et al., 1990) and these are thought to be due to differences in the conformational features of the collagen molecules rather than in their primary sequences (Mallya et al., 1990; Netzel-Arnet et al., 1991). However, little is known about the differences in specificity for substrates other than the collagens (Murphy et al., 1992).

Collagenase fragments that retain proteolytic activity are derived from autolytic cleavage between the N- and C-terminal portions of the enzymes. This fragmentation occurs naturally *in vitro* and has been described for both collagenases (Birkedal-Hansen et al., 1988; Clark and Cawston, 1989; Knäuper et al., 1993). The isolated N-terminal fragment retains its proteolytic activity against casein substrates, but is no longer able to cleave collagen, suggesting that the C-terminal domain is essential for collagen specificity. More recent studies using recombinant collagenase–stromelysin hybrids have shown that both the N-terminal and C-terminal domains contribute towards collagenase specificity (Murphy et al., 1992).

Aggrecan is the predominant structural proteoglycan present in cartilage, and the accelerated loss of aggrecan in arthritic disease is a major cause of cartilage failure. In this paper we describe the actions of fibroblast and neutrophil collagenases against human and porcine aggrecan and identify specific cleavage sites made by them within the proteinase-sensitive inter-

Abbreviation used: PMN, polymorphonuclear.
¶ To whom correspondence should be addressed.

globular domain. We have also investigated the actions of collagenase catalytic fragments. The results show that the specificities for cartilage aggrecan of fibroblast and neutrophil collagenases, and of their corresponding catalytic fragments, are identical to each other, but different from those observed for the stromelysins and gelatinases.

MATERIALS AND METHODS

$Na^{125}I$ (IMS 30) and ECL reagents were from Amersham (Australia). Trypsin (diphenylcarbamoyl chloride-treated) (EC 3.4.21.4), soybean trypsin inhibitor, 1,10-phenanthroline, 3-dimethylaminopropionitrile, 6-aminohexanoic acid, phenylmethanesulphonyl fluoride, benzamidine hydrochloride, 4-aminophenylmercuric acetate and biotinylated anti-rabbit immunoglobulin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Agarose type HSC was from PS Park Scientific (Northampton, U.K.). Vectorstain ABC kit was from Immunodiagnostics (Camperdown, NSW, Australia). A Bio-Sil SEC-400 h.p.l.c. column was from Bio-Rad (Sydney, Australia). Human umbilical chord hyaluronan was from BDH (Poole, Dorset, U.K.). Immobilon was from Millipore–Waters (Sydney, Australia). All other reagents were of analytical grade except for guanidine hydrochloride (Sigma), which was purified with activated charcoal and diatomaceous earth (Sigma).

The preparations of aggrecan (Hardingham, 1979; Bayliss and Roughley, 1985), aggrecan G1–G2 fragments (Fosang and Hardingham, 1989), specific rabbit antisera against G1 and G2 core protein domains (Ratcliffe and Hardingham, 1983; Fosang and Hardingham, 1989) and recombinant human prostromelysin (Docherty and Murphy, 1990; Koklitis et al., 1991) have been described previously.

Preparation and activation of enzymes

Recombinant human fibroblast procollagenase was expressed in NSO cells as described previously (Murphy et al., 1992). Enzyme activity was approx. 14225 units/mg when determined by the degradation of soluble type I collagen at 25 °C and gel scanning (Welgus et al., 1981a). Human polymorphonuclear (PMN) leucocyte procollagenase was purified to homogeneity as previously described (Knäuper et al., 1990), and identity was confirmed by comparison of the N-terminal sequence of the proenzyme with the sequence of the cDNA clone (Hasty et al., 1990a). The enzyme had a specific activity of 29096 units/mg, as determined by gel scanning. Purified fibroblast collagenase catalytic fragment (lacking the C-terminal domain) was kindly provided by Dr. I. Clark and Dr. T. Cawston (Clark and Cawston, 1989), and contained 200 units/mg caseinase activity. Purified PMN leucocyte collagenase catalytic fragment (40 kDa) was prepared by dialysing affinity-purified active PMN leucocyte collagenase (60–100 μg/ml) against 20 mM Tris/HCl, pH 7.5, 10 mM $CaCl_2$ and 0.5 M NaCl prior to incubation for 24 h at 37 °C to allow fragmentation. The fragments were separated using a hydroxamic acid Sepharose column (1.8 cm × 18 cm) (Moore and Spilburg, 1986). Recombinant PMN collagenase catalytic domain fragment was purified using the same protocol.

Enzyme digestions

The proenzymes (10 μg) were activated by incubation with 0.1 μg of recombinant human prostromelysin and 0.5 μg of trypsin for 30 min at 37 °C in a total volume of 50 μl. Trypsin was inactivated with 5 μg of soybean trypsin inhibitor. Control tubes containing prostromelysin and trypsin did not degrade aggrecan or G1–G2 substrates. Samples for sequencing were digested with enzymes that had been activated with 1 mM $HgCl_2$ for 1 h at 37 °C (Blaser et al., 1991), and gave identical fragmentation patterns on SDS gels. Enzyme digestions were carried out in buffer containing 10 mM $CaCl_2$, 100 mM NaCl and 50 mM Tris/HCl, pH 7.5, at 37 °C and the digests were stopped by the addition of EDTA and 1,10-phenanthroline to 10 mM and 2 mM final concentrations respectively. ^{125}I-labelled G1–G2 digestion products were analysed on 5% SDS gels (Fairbanks et al., 1971). Aggrecan digestion products were analysed on polyacrylamide/agarose composite gels (Carney et al., 1986).

Purification of collagenase-derived G2 products and N-terminal sequencing

G2 fragments derived from collagenase digests were isolated by size exclusion chromatography on a Bio-Sil SEC 400 h.p.l.c. column after mixing overnight at 4 °C with hyaluronan, in order to bind all G1 fragments and exclude them in the void of the column (Fosang and Hardingham, 1989; Fosang et al., 1992). Column fractions were analysed by electrophoresis on 5% SDS gels (Fairbanks et al., 1971), and the fractions containing high- or low-molecular-mass G2 fragments were pooled separately, dialysed and freeze-dried for N-terminal sequencing as described previously (Sandy et al., 1990).

RESULTS AND DISCUSSION

Collagenase digestion of aggrecan

Human articular and pig laryngeal aggrecan were digested with fibroblast and neutrophil collagenases and analysed by dissociative composite gel electrophoresis. Samples of undigested human aggrecan contained three distinct bands (Bayliss, 1992) (Figure 1, lane 1), while digested fragments migrated on gels with faster relative electrophoretic mobilities (Figure 1, lanes 2–5). Undigested pig aggrecan migrated as a single species (Figure 1, lane 6), and had a slower relative mobility than human aggrecan. Both collagenases were able to degrade pig aggrecan into faster-migrating products (Figure 1, lanes 7–10). Based on the results of digestions at two different enzyme concentrations and the relative mobilities of the digestion products, it appears that the specific activities of both collagenases for aggrecan are similar.

Collagenase digestion of aggrecan G1–G2

An aggrecan G1–G2 fragment has been used to investigate cleavage occurring between the G1 and G2 globular protein

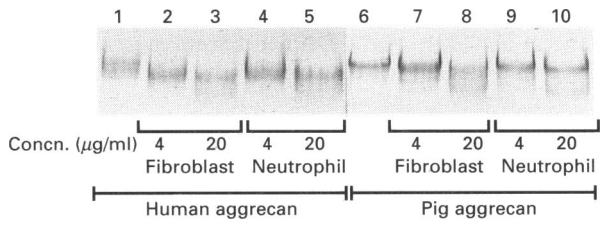

Figure 1 Collagenase digestion of human and porcine aggrecans

Aliquots of samples containing human aggrecan (32 μg; lanes 1–5) and pig aggrecan (25 μg; lanes 6–10) were electrophoresed on polyacrylamide/agarose composite gels before (lanes 1 and 6) and after digestion with either fibroblast collagenase (4 μg/ml, lanes 2 and 7; 20 μg/ml, lanes 3 and 8) or neutrophil collagenase (4 μg/ml, lanes 4 and 9; 20 μg/ml, lanes 5 and 10). Samples were digested for 18 h at 37 °C in a total volume of 50 μl.

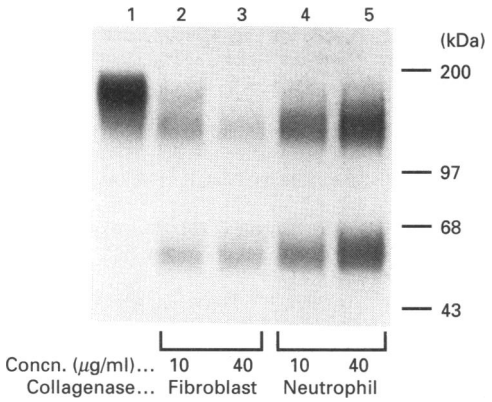

Figure 2 Digestion of ^{125}I-labelled aggrecan G1–G2

Purified ^{125}I-labelled G1–G2 was analysed by SDS/PAGE and autoradiography before (lane 1) or after digestion with either fibroblast collagenase (10 μg/ml and 40 μg/ml, lanes 2 and 3 respectively) or neutrophil collagenase (10 μg/ml and 40 μg/ml, lanes 4 and 5 respectively). Samples were digested for 18 h at 37 °C in a total volume of 20 μl. The small differences in signal intensity in lanes 2 and 3 are probably due to lower loading.

domains (Fosang et al., 1991). When digested with fibroblast or neutrophil collagenase, ^{125}I-labelled G1–G2 (150 kDa) gave products of 56 kDa and 110 kDa (Figure 2). These were of a similar size to those produced by other metalloproteinases (Fosang et al., 1991, 1992). However, results from immunolocalization studies with specific anti-G1 and anti-G2 antisera showed that although the G1 domain was located only in the 56 kDa band, the G2 domain was present in both the 110 kDa and 56 kDa products. This is compatible with two cleavage sites, as was previously found with the low-molecular-mass metalloproteinase matrilysin (Fosang et al., 1992), but different from the stromelysins and gelatinases that cleave at only a single site. The low- (56 kDa) and high- (110 kDa) molecular-mass products containing the G2 domain were resolved from each other by size-exclusion chromatography, and at the same time separated from fragments containing G1 which were bound to added hyaluronan and eluted in the void volume of the column. N-terminal sequencing of the G2-containing fragments showed the 110 kDa product to be cleaved at Asn^{341}-Phe^{342} and the 56 kDa product to be cleaved at Asp^{441}-Leu^{442} (Figure 3). The products were thus identical to those previously identified in matrilysin digests and, as with matrilysin, the results with collagenase show that cleavage at the Asn^{341}-Phe^{342} site is more complete than at the Asp^{441}-Leu^{442} site, otherwise no 110 kDa product containing G2 would be produced. Cleavage at Asn^{341}-Phe^{342} therefore appears to be more favoured than that at Asp^{441}-Leu^{442}.

Collagenase catalytic fragments lacking the C-terminal hemopexin-like domain were tested for their actions against aggrecan G1–G2. These truncated enzymes are more similar to matrilysin, which also lacks the C-terminal domain. This domain is thought to be involved in the binding of metalloproteinases to triple-helical collagen (Allan et al., 1991; Murphy et al., 1992), and while it is essential for the specificity of collagenases for collagen (Birkedal-Hansen et al., 1988; Clark and Cawston, 1989; Schnierer et al., 1993), it does not appear to be essential for the specificity of collagenases for non-collagen substrates (Murphy et al., 1992), or for stromelysin activity (Okada et al., 1986; Koklitis et al., 1991). Thus collagenase catalytic fragments were used to determine whether the C-terminal domain has any influence on the specificity of the collagenases for aggrecan. The results showed that, compared with the full-length enzymes, identical digestion products were produced following incubation with either a purified fibroblast collagenase catalytic fragment, or native and recombinant neutrophil collagenase catalytic fragments (Table 1). Loss of the C-terminal domain of the collagenases did not, therefore, have a role in determining the specificity of the enzymes for either of the two cleavage sites in the aggrecan interglobular domain.

Together with our previous findings, the present results show that all of the known mammalian matrix metalloproteinases cleave aggrecan at a predominant site between Asn^{341} and Phe^{342} (human and rat sequences). In addition, fibroblast and neutrophil collagenases, and matrilysin, make a second cleavage in the interglobular domain near to G2, liberating a fragment of approx. 100 amino acids that is substituted with several keratan sulphate

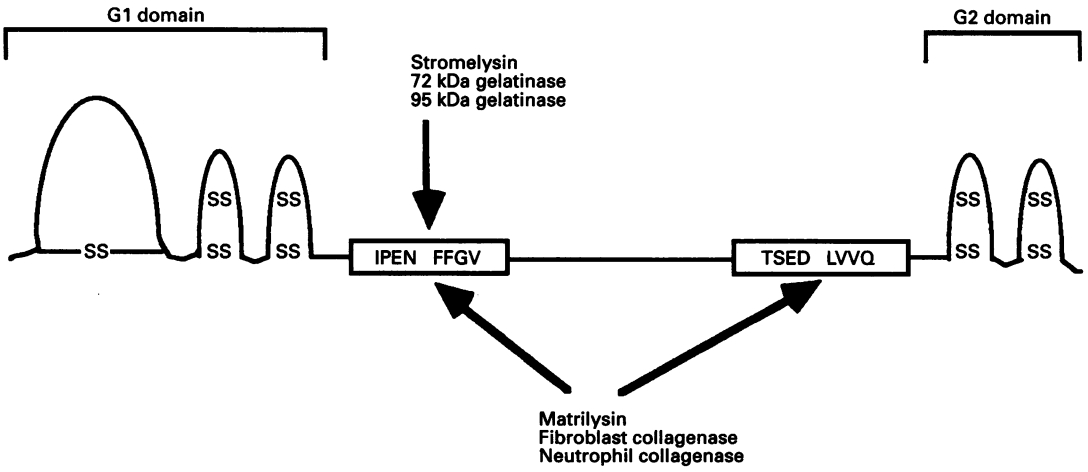

Figure 3 Metalloproteinase cleavage sites in the aggrecan interglobular domain

Schematic representation of the N-terminal G1 and G2 domains of cartilage aggrecan, and the interglobular domain between them. The specific sequences, and the relative locations of the sites recognized by the mammalian matrix metalloproteinases, are shown. SS represents disulphide bonds, and the amino acid sequences are given by the single-letter code.

Table 1 N-terminal sequence analysis of the G2-containing products of collagenase digests

G2-containing fragments (110 kDa and 56 kDa) present in collagenase digestions were purified by size-exclusion h.p.l.c. and analysed by N-terminal sequencing. The initial yields were between 30 and 100 pmol. The identities of residues 5 and 6 in the lower G2-derived sequence are equivocal but are likely to be V and T respectively, based on similarities with human and rat sequences.

Enzyme	N-terminal sequences
Recombinant fibroblast collagenase	FFGVGGXEDIXIQTVXWPDVE XXXQEPMAPGAV
Fibroblast collagenase fragment	FFGVGGEEDIXIQTV XXXQVPMAPGA
Neutrophil collagenase	FFGVGGEEDIXIQTX LVVQVTMAPGA
Native neutrophil collagenase fragment	FFGVGGEEDIXIQTV LVVQVTMAPGAV
Recombinant neutrophil collagenase fragment	FFGVGGEEDITIQTV LVVQVTMA

chains (Fosang et al., 1992). While there is evidence for proteolytic processing of aggrecan at the predominant metalloproteinase site *in vivo*, there is no evidence to suggest that cleavage at the second site occurs in tissues.

The reason why matrilysin and the collagenases cleave at a site in G1–G2 that is not susceptible to other metalloproteinases is unclear. It is likely that the conformation around the cleavage site, and possibly also adjacent glycosylation, is important in determining the specificity of these metalloproteinases for aggrecan. It is clear, however, that the C-terminal domain of metalloproteinases is not essential for the recognition of these cleavage sites in aggrecan, because truncated enzymes such as matrilysin and the collagenase catalytic fragments cleave at the same Asn-Phe site as the other metalloproteinases.

It is not known whether collagenase catalytic fragments capable of degrading aggrecan, but not collagen, are generated in cartilage, or indeed in any tissues. The possibility that they may occur naturally *in vivo* has important physiological implications for a tissue like cartilage, the biomechanical properties of which are dependent on the integrity of an extracellular matrix where type II collagen and aggrecan are the major structural components.

This work was supported by the Victorian Health Promotion Foundation (Australia), the Arthritis Foundation of Australia, the National Health and Medical Research Council (Australia), the Arthritis and Rheumatism Council (U.K.) and the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, Germany. We thank Carmen Young for technical expertise and Thomas Kleine for cloning and expression of the neutrophil collagenase catalytic fragment.

REFERENCES

Allan, J. A., Hembry, R. M., Angal, S., Reynolds, J. J. and Murphy, G. (1991) J. Cell Sci **99**, 789–795

Bayliss, M. T. (1992) in Articular Cartilage and Osteoarthritis (Kuettner, K. E., Schleyerbach, R., Peyron, J. G. and Hascall, V. C., eds.), pp. 487–500, Raven Press, New York.

Bayliss, M. T. and Roughley, P. J. (1985) Biochem. J. **232**, 111–117

Birkedal-Hansen, B., Moore, W. G. I., Taylor, R. E., Bhown, A. S. and Birkedal-Hansen, H. (1988) Biochemistry **27**, 6751–6758

Blaser, J., Knäuper, V., Osthues, A., Reinke, H. and Tschesche, H. (1991) Eur. J. Biochem. **202**, 1223–1230

Carney, S. L., Bayliss, M. T., Collier, J. M. and Muir, H. (1986) Anal. Biochem. **156**, 38–44

Clark, I. M. and Cawston, T. E. (1989) Biochem. J. **263**, 201–206

Dean, D., Azzo, W., Martel-Pelletier, J., Pelletier, J.-P., Howell, D. S. and Woessner, J. F., Jr. (1987) J. Rheumatol. **14** (Suppl.), 43–44

Docherty, A. J. P. and Murphy, G. (1990) Ann. Rheum. Dis. **49**, 469–479

Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry **10**, 2606–2616

Fosang, A. J. and Hardingham, T. E. (1989) Biochem. J. **261**, 801–809

Fosang, A. J., Neame, P. J., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1991) J. Biol. Chem. **266**, 15579–15582

Fosang, A. J., Neame, P. J., Last, K., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1992) J. Biol. Chem. **267**, 19470–19474

Hardingham, T. E. (1979) Biochem. J. **177**, 237–247

Hasty, K. A., Stricklin, G. P., Hibbs, M. S., Mainardi, C. L. and Kang, A. H. (1987) Arthritis Rheum. **30**, 695–699

Hasty, K. A., Pourmotabbed, T. F., Goldberg, G. I., Thompson, J. P., Spinella, D. G., Stevens, R. M. and Mainardi, C. L. (1990a) J. Biol. Chem. **265**, 11421–11424

Hasty, K. A., Reife, R. A., Kang, A. H. and Stuart, J. M. (1990b) Arthritis Rheum. **33**, 388–397

Hughes, C., Murphy, G. and Hardingham, T. E. (1991) Biochem. J. **279**, 733–739

Knäuper, V., Kramer, S., Reinke, H. and Tschesche, H. (1990) Eur. J. Biochem. **189**, 295–300

Knäuper, V., Osthues, A., DeClerck, Y. A., Langley, K. E., Blaser, J. and Tschesche, H. (1993) Biochem. J. **291**, 847–854

Koklitis, P. A., Murphy, G., Sutton, C. and Angal, S. (1991) Biochem. J. **276**, 217–221

Lohmander, L. S., Hoerrner, L. A. and Lark, M. W. (1993) Arthritis Rheum. **36**, 181–189

Mallya, S. K., Mookhtiar, K. A., Gao, Y., Brew, K., Dioszegi, M., Birkedal-Hansen, H. and Van Wart, H. E. (1990) Biochemistry **29**, 10628–10634

Martel-Pelletier, J., Pelletier, J.-P., Cloutier, J.-M., Howell, D. S., Ghandur-Mnaymneh, L. and Woessner, J. F., Jr. (1984) Arthritis Rheum. **26**, 63–68

Mitchell, P. G. and Cheung, H. S. (1991) J. Cell. Physiol. **149**, 132–140

Moore, W. M. and Spilburg, C. A. (1986) Biochemistry **25**, 5189–5195

Murphy, G., Hembry, R. M. and Reynolds, J. J. (1986) Collagen Relat. Res. **6**, 351–364

Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. I., O'Connell, J. P. and Docherty, A. J. P. (1992) J. Biol. Chem. **267**, 9612–9618

Netzel-Arnet, S., Fields, G., Birkedal-Hansen, H. and Van Wart, H. E. (1991) J. Biol. Chem. **266**, 6747–6755

Okada, Y., Nagase, H. and Harris, E. D. (1986) J. Biol. Chem. **261**, 14245–14255

Okada, Y., Shinmei, M., Tanaka, O., Naka, K., Kimura, A., Nakanishi, I., Bayliss, M. T., Iwata, K. and Nagase, H. (1992) Lab. Invest. **66**, 680–690

Pelletier, J.-P. and Martel-Pelletier, J. (1985) Arthritis Rheum. **28**, 1393–1401

Pelletier, J.-P., Martel-Pelletier, J., Altman, R. D., Ghandur-Mnaymneh, L., Howell, D. S. and Woessner, J. F., Jr. (1983) Arthritis Rheum. **26**, 866–874

Ratcliffe, A. and Hardingham, T. E. (1983) Biochem. J. **213**, 371–378

Richards, C. D., Rafferty, J. A., Reynolds, J. J. and Saklatvala, J. (1991) Matrix **11**, 161–167

Sandy, J. D., Flannery, C. R., Boynton, R. E. and Neame, P. J. (1990) J. Biol. Chem. **265**, 21108–21113

Schnierer, S., Kleine, T., Gote, T., Hillemann, A., Knäuper, V. and Tschesche, H. (1993) Biochem. Biophys. Res. Commun. **191**, 319–326

Walakovits, L. A., Moore, V. L., Bhardwaj, N., Gallick, G. S. and Lark, M. (1992) Arthritis Rheum. **35**, 35–42

Welgus, H. G., Jeffrey, J. J. and Eisen, A. Z. (1981a) J. Biol. Chem. **256**, 9516–9521

Welgus, H. G., Jeffrey, J. J. and Eisen, A. Z. (1981b) J. Biol. Chem. **256**, 9511–9515

Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H.-J., Smith, B. J., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P. and Docherty, A. J. P. (1986) Biochem. J. **240**, 913–916

Wilhelm, S. M., Eisen, A. Z., Teter, M., Clark, S. D., Kronberger, A. and Goldberg, G. I. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 3756–3760

Received 4 March 1993/10 May 1993; accepted 20 May 1993