Activation of precursors for matrix metalloproteinases 1 (interstitial collagenase) and 3 (stromelysin) by rat mast-cell proteinases I and II

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Histological studies have previously demonstrated an association between mast-cell activation/degranulation and areas of connective-tissue lysis in vivo; in addition, mast-cell extracts have been shown to activate latent forms of collagenase and stromelysin. In the present study we have examined the potential roles of rat mast-cell proteinase (RMCP) I and RMCP II as activators of the precursors of matrix metalloproteinase (MMP)-1 (interstitial collagenase), MMP-2 (gelatinase A) and MMP-3 (stromelysin-1). Both RMCPs I and II activated proMMP-3 by converting the 57 kDa precursor into a 45 kDa polypeptide. The N-terminal amino acid of 45 kDa MMP-3 activated by RMCP II was identified as Phe81. By contrast, only RMCP II activated the 52 kDa proMMP-1 by converting it into a 41 kDa protein and generating the new N-termini, namely Glu112 and Val119. The collagenolytic activity which resulted from this cleavage was only 35% of the full activity; but this could not be augmented by subsequent treatment with MMP-3, the latter being a crucial enzyme for the generation of the fully active MMP-1 with Phe112 at the N-terminus, in conjunction with other serine proteinases. Thus RMCP II activates proMMP-1 via a mechanism different from that reported for the stepwise processing by combinations of other trypsin-like enzymes and MMP-3. ProMMP-2 (progelatinase A) was not activated by either RMCP I or RMCP II, despite processing to smaller products.

INTRODUCTION

Matrix metalloproteinases (MMPs) are considered to play central roles in the degradation of connective tissues (Woessner, 1991; Birkedal-Hansen et al., 1993). Three main MMPs secreted from rheumatoid synovial cells and other connective-tissue cells have been isolated and characterized for their abilities to degrade extracellular-matrix components. MMP-1 (interstitial collagenase) attacks collagen types I, II, III, VII and X (Seltzer et al., 1989; Schmid et al., 1986); in addition, it is capable of degrading casein (Windsor et al., 1991) and cartilage proteoglycan (Hughes et al., 1991). MMP-2 (gelatinase A) readily degrades gelatins, but also has activity on collagen types IV, V and VII, elastin and proteoglycan (Murphy et al., 1985, 1991; Seltzer et al., 1989; Okada et al., 1990; Senior et al., 1991). MMP-3 (stromelysin-1) has a wide spectrum of substrates that include proteoglycans, laminin, fibronectin and collagen types IV and IX (Okada et al., 1986, 1989). All three enzymes are secreted from the cell as precursor forms which require activation. The activation of proMMP-1 can be initiated by trypsin-like serine proteinases to generate partially active MMP-1 intermediates, but the action of MMP-3 is crucial for the subsequent full activation of MMP-1 (Murphy et al., 1987; Ito and Nagase, 1988; He et al., 1989; Suzuki et al., 1990). The activation of proMMP-3 can be achieved by various serine proteinases such as trypsin, chymotrypsin, human neutrophil elastase, plasmin, and plasma kallikrein (Okada et al., 1988) by stepwise processing of the propeptide (Nagase et al., 1990). All thesezymogens are also activated in vitro by exposure to organomercurials (Woessner, 1991). However, the mechanism(s) of metalloproteinase activation in vivo remains uncertain. It is likely that these activator enzymes may arise from the connective-tissue cell itself or from other cell types such as granulocytes and macrophages.

Several reports have noted the association of mast cells with areas of connective-tissue degradation, especially at sites of tumour invasion in breast carcinoma and melanoma (Hartveit et al., 1984; Dabbous et al., 1986) and at sites of cartilage erosion in rheumatoid arthritis (Bromley et al., 1984; Bromley and Woolley, 1984). Mast-cell proteinase extracts were reported to activate proMMP-1 (Birkedal-Hansen et al., 1976; Dabbous et al., 1986), and recent studies have shown that mast-cell tryptase generates collagenase activity indirectly through its ability to activate proMMP-3 (Gruber et al., 1989). In addition to tryptase, mast cells may contain chymases, serine proteinases which are chymotrypsin-like in substrate specificity and molecular mass (Katunuma et al., 1975; Woodbury and Neurath, 1978; Woodbury et al., 1981). These enzymes have been characterized in rat and designated as rat mast-cell proteinase I (RMCP I) and II (RMCP II) (Woodbury and Neurath, 1978; Woodbury et al., 1981). RMCP I is the major proteinase component of connective-tissue mast cells, whereas RMCP II is derived from mucosal mast cells (Woodbury et al., 1978a,b). Similar enzymes are present in human mast cells, and the content of tryptase relative to chymase is one of the main features of mast-cell heterogeneity, which is recognized not only between species, but also between different tissues (Irani et al., 1986; Weidner and Austin, 1993). Since chymases have different substrate specificities compared with tryptase, we have examined the ability of RMCP I and II to activate the precursor forms for MMP-1, -2 and -3. Our observations not only indicate that mast-cell chymases are potential activators for some members of the MMP family, but also demonstrate differences in the enzymic properties of RMCP I and RMCP II.

Abbreviations used: APMA, 4-aminophenylmercuric acetate; DFP, di-isopropyl fluorophosphate; MMP, matrix metalloproteinase; RMCP, rat mast-cell proteinase; Tos-Lys-CH2Cl, tosyl-lysylchloromethane ("TLCK"); Cm-Tf, carboxymethylated transferrin.

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Materials and Methods

Materials
4-Aminophenylmercuric acetate (APMA), Brij 35, tosyl-lysyl-chloromethane (Tos-Lys-CH₂Cl; ‘TLCK’), were from Sigma Chemical Co. Protein standards for SDS/PAGE were from Bio-Rad Laboratories.

Enzymes
ProMMP-1 (interstitial procollagenase), proMMP-2 (progelatinase A) and proMMP-3 (stromelysin 1) were purified from culture medium of human rheumatoid synovial fibroblasts stimulated with rabbit macrophage-conditioned medium as described previously (Suzuki et al., 1990; Okada et al., 1990; Ito and Nagase, 1988). The final products were homogeneous on SDS/PAGE. RMCP I was purified from mast cells obtained from rat peritoneal lavage and RMCP II from the small intestine of rats hyperimmunized with the nematode Nippostrongylus brasiliensis as described previously (Gibson et al., 1987). The final products were homogeneous on SDS/PAGE. However, since a very small amount of trypsin-like activity was detected in the RMCP II preparation this was eliminated by treatment with 100 μM Tos-Lys-CH₂Cl at 23 °C for 1 h prior to use in proMMP activation studies.

Enzyme assays
Collagenolytic activity was measured by the diffuse fibril method of Cawston and Barrett (1979) using [¹⁴C]acetylated type I collagen (guinea-pig) at 37 °C. Gelatinolytic activity was measured by using heat-denatured [¹⁴C]acetylated type I collagen and 20% (w/v) trichloroacetic acid to precipitate undegraded substrate (Vater et al., 1983). One unit of collagenolytic or gelatinolytic activity was defined as 1 μg of collagen or gelatin digested/min at 37 °C. MMP-3 activity was measured using reduced [³H]carboxymethylated transferrin ([³H]Cm-Tf) as described by Okada et al. (1986). One unit of MMP-3 was defined as the production of 1 μg of Cm-Tf fragments soluble in 3.3% (w/v) trichloroacetic acid in 1 min at 37 °C. All the enzyme assay and activation studies were carried out in 50 mM Tris/HCl buffer (pH 7.5)/0.15 M NaCl/10 mM CaCl₂/0.02% NaN₃/0.05% Brij 35.

Electrophoresis
SDS/PAGE was performed by the method of Bury (1981). After electrophoresis the proteins were stained with AgNO₃ (Wray et al., 1981) or Coomassie Brilliant Blue R250. The molecular-mass standards used were phosphorylase b (94 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa).

Western-blot analysis
Samples resolved by SDS/PAGE (7.5% total acrylamide) under reducing conditions were electrotransferred on to nitrocellulose filters as described by Burnett (1981). Non-specific binding of IgG was blocked by 22% (w/v) fat-free-milk solution. The filters were then allowed to react with sheep anti-(human MMP-3) serum (1:3000 dilution) at room temperature for 16 h. After extensive washing, anti-MMP-3 antibodies bound to the antigens were allowed to form a complex with peroxidase-conjugated goat anti-(sheep IgG) IgG for 2 h at room temperature and the protein bands were subsequently revealed using diaminobenzidine tetrahydrochloride and H₂O₂ (Towbin et al., 1979).

Figure 1 Activation of proMMP-3 by RMCP I and RMCP II
ProMMP-3 (5 μg/ml) was incubated with (a) RMCP I and (b) RMCP II at 37 °C for various periods of time at concentrations of 1 μg/ml (△) and 5 μg/ml (○). After inactivating RMCP I and II with 2 mM di-isopropyl fluorophosphate (DFP), MMP-3 activity was measured against [³H]Cm-Tf. Activation of proMMP-3 by 1.5 mM APMA (●) and proMMP-3 without activation (×) are shown for comparison in (a) and (b) respectively.

N-terminal sequence analysis
Automatic sequence analyses were performed with an Applied Biosystems 477A pulse liquid-phase sequencer with ‘on line’ 120A phenylthiolydantoin-amino acid analysis. To determine the N-terminal sequence, RMCP II-treated proMMP-1 or proMMP-3 was separated by SDS/PAGE (7.5% total polyacrylamide) under reducing conditions and transferred from the polyacrylamide gel to poly(vinylidene difluoride) membrane according to the procedure described by Matsudaira (1987). The proteins transferred to the poly(vinylidene difluoride) membrane were located by staining with Coomassie Brilliant Blue R250, and the bands of interest were excised, placed on a Polybrene-treated glass filter and sequenced.

Results
Activation of ProMMP-3 by RMCPs I and II
Incubation of proMMP-3 with RMCP I at 37 °C resulted in concentration- and time-dependent activation: the precursor was fully activated by 5 μg/ml of RMCP I within 1 h. This activation rate was about three times higher than that observed with 1.5 mM APMA at 37 °C (Figure 1a). Incubation with RMCP II at 37 °C also activated in a concentration- and time-dependent manner, but the rate of activation by RMCP II was lower than that by RMCP I (Figure 1b). The specific activity of the fully activated MMP-3 was 845 units/mg.

SDS/PAGE analysis of proMMP-3 activated by RMCP I or RMCP II showed that proMMP-3 was converted first into the active form of 45 kDa, and then converted into a 28 kDa polypeptide on longer incubation (Figure 2), a response typically seen after an incubation with APMA (Nagase et al., 1990). Both the 45 kDa and the 28 kDa species were previously shown to have indistinguishable proteolytic activity (Okada et al., 1986).
N-terminal sequence of RMCP II-activated 45 kDa MMP-3 was Phe-Arg-Thr-Phe-Pro, identical with the sequence produced by human neutrophil elastase or APMA (Nagase et al., 1990). These results suggest that both RMCPs I and II follow a typical stepwise activation of proMMP-3 as described for other proteinases (Nagase et al., 1991).

Activation of proMMP-1 by RMCP II

Incubation of proMMP-1 with RMCP I (1–5 μg/ml) at 37 °C up to 72 h did not exhibit collagenolytic activity. However, when proMMP-1 was incubated with RMCP II the activity against type I collagen increased in a time-dependent manner, but the maximal specific activity of MMP-1 was limited to about 35% of the fully active MMP-1 (9450 compared with 27000 units/mg). Since MMP-3 is required for full activation of proMMP-1 (Suzuki et al., 1990), the proMMP-1 treated with RMCP II was allowed to react further with MMP-3. However, the specific activity of MMP-1 did not increase, whereas proMMP-1 treated with plasmin or APMA was fully activated by subsequent incubation with MMP-3 (see Table 1).

SDS/PAGE analysis showed that proMMP-1 was converted into a 41 kDa protein by RMCP II (Figure 3). Further treatment of this 41 kDa MMP-1 with MMP-3 did not cause any changes in molecular mass (Figure 3). Similarly, proMMP-1 co-treated with RMCP II and MMP-3 did not show any significant changes in the specific activity and molecular mass (results not shown). These results differ with those obtained after plasmin treatment of proMMP-1; plasmin initially processed the 52 kDa proMMP-1 to a 46 kDa protein, then to a 43 kDa MMP-1 which exhibited 14% of the full activity (Table 1). The 43 kDa form was further converted into the fully active 41 kDa MMP-1 by the specific action of MMP-3 (Table 1 and Figure 3). During the treatment of proMMP-1 with RMCP II there was no 43 kDa species detected.

Table 1 Activation of proMMP-1 by RMCP II

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<tr>
<th>Treatment</th>
<th>Reaction time (h)</th>
<th>Collagenolytic activity (%)</th>
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<td></td>
<td></td>
<td>− MMP-3</td>
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<tr>
<td>proMMP-1</td>
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<td>0</td>
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<tr>
<td></td>
<td>72</td>
<td>80</td>
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<tr>
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<tr>
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<td>3770</td>
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Figure 2 Processing of proMMP-3 by RMCPs I and II

ProMMP-3 (5 μg/ml) was treated with RMCP I (5 μg/ml) or RMCP II (5 μg/ml) at 37 °C for various periods of time. The reactions were terminated with 2 mM DFP and 20 mM EDTA and the samples analysed by SDS/PAGE under reducing conditions by silver staining (lanes 1–6) or Western blot (lanes 7 and 8). Lane 1, proMMP-3 alone; lanes 2–6, proMMP-3 incubated with RMCP I at 37 °C for 30 min, 1, 2, 4 and 24 h respectively; lanes 7 and 8, proMMP-3 treated with RMCP II at 37 °C for 4 and 24 h respectively.

Effect of RMCP I and RMCP II on proMMP-2

Treatment of proMMP-2 with RMCP I resulted in reduction in size from 72 kDa to a 50 kDa polypeptide within 10 min, and then further conversion into 41 kDa and 29 kDa species (results not shown), but no gelatinolytic activity was detected. Similar results were obtained with RMCP II. Treatment of proMMP-2 with APMA initially generated 68 kDa and then 41 kDa proteins, which were further processed to smaller fragments; the generation of the 68 kDa MMP-2 correlated with the expression of...
gelatinolytic activity of MMP-2 as described previously (Okada et al., 1990). Such a molecular species was not observed after incubation with RMCP I or RMCP II.

**DISCUSSION**

MMPs secreted from connective-tissue cells are key enzymes in degradation of extracellular matrix under both physiological and pathological conditions [see Woessner (1991), Matrisian (1992) and Birkedal-Hansen et al. (1993) for reviews]. The production of proMMP-1 and proMMP-3 from mesenchymal cells such as fibroblasts and chondrocytes is greatly enhanced by inflammatory cytokines and other stimulatory factors (see Woessner, 1991; Birkedal-Hansen et al., 1993), but once secreted thesezymogens require local activation to express their enzymic properties. Observations of mast-cell activation associated with connective-tissue degradation (Bromley et al., 1984; Dabbous et al., 1986) have suggested that mast-cell proteins may contribute to matrix breakdown, possibly by their potential to activate proMMPs at these tissue sites. In the present study we have examined two closely related RMCPs, I and II, for their abilities to activate proMMPs -1,-2 and -3, and have demonstrated that both enzymes activate proMMP-3, but only RMCP II participates in proMMP-1 activation. Neither proteinase activated proMMP-2.

Our previous studies showed that a large number of proteinases, regardless of their specificities, could activate proMMP-3, but activation of proMMP-1 was restricted to trypsin-like enzymes (Nagase et al., 1991). This is indicated by sequences around the ‘bait’ region located in the middle of MMP propeptides where the initial proteolytic cleavages take place. In proMMP-3 this region was identified in the stretch Phe$^{44}$-Val$^{45}$-Arg$^{46}$-Lys$^{47}$ that accommodates activating cleavages by a number of proteinases (Nagase et al., 1990). The intermediate forms thus generated react with each other and are converted into the fully active 45 kDa MMP-3 by cleavage of the His$^{44}$-Phe$^{45}$ bond at the junction of the propeptide and catalytic domain (Nagase et al., 1990). Since chymotrypsin cleaves the Phe$^{44}$-Val$^{45}$ bond, it was conceivable that both RMCPs I and II would attack this bond and thereby trigger the stepwise activation of proMMP-3. Indeed, the N-terminal sequence of the final 45 kDa MMP-3 generated with RMCP II revealed Phe$^{44}$ as its N-terminus, as predicted from the previous studies with other proteinases (Nagase et al., 1990).

The activation of proMMP-1 by the chymotrypsin-like proteinase RMCP II was unexpected, since previous studies suggested that the initial cleavage of the ‘bait region’ of proMMP-1 (Glu$^{42}$-Lys-Arg-Arg-Asn$^{47}$) is required for activation. This generation of intermediate forms is further processed to the 43 kDa or the 41 kDa form by autolysis, or to the fully active 41 kDa collagenase by specific cleavage of the Gln$^{46}$-Phe$^{47}$ bond by MMP-3 (Suzuki et al., 1990). The lack of aromatic residues in this region suggests that RMCP II may directly attack the Ala$^{42}$-Gln$^{46}$ and the Phe$^{46}$-Val$^{47}$ bonds. Indeed, intermediate forms (43–47 kDa) of MMP-1 were not observed with RMCP II even after incubation at 0°C for 48 h, conditions under which autolytic processing of MMP-1 was largely suppressed (results not shown). Thus the mode of proMMP-1 activation by RMCP II appears to by-pass the stepwise processing of the propeptide demonstrated for other activating proteinases. While this paper was under review, Saarinen et al. (1994) reported that human mast-cell chymase also directly activates human proMMP-1 by cleaving the Leu$^{43}$-Thr$^{44}$ bond, and this activation process was markedly enhanced by heparin. However, RMCP II activity is not dependent on the presence of heparin. The different cleavage sites in proMMP-1 produced by human chymase (Saarinen et al., 1994) and RMCP II may result from subtle differences in substrate requirements of the two enzymes.

Incubation of proMMP-1 with RMCP II resulted in about 35% of the full collagenolytic activity, but the activity did not increase after incubation with MMP-3. This observation is analogous to that of MMP-1 generated by autolytic cleavage of the Phe$^{43}$-Val$^{44}$ and the Val$^{44}$-Leu$^{45}$ bonds at the junction of the propeptide and catalytic domain of MMP-1 (Suzuki et al., 1990).
Autolytically generated MMP-1 with N-terminal Val\textsuperscript{88} or Leu\textsuperscript{88} expressed only about 40\% of the maximal collagenolytic activity. These observations have led us to postulate that N-terminal Phe\textsuperscript{38} plays a critical role in governing the level of collagenolytic activity. The importance of Phe\textsuperscript{38} of the N-terminus for the full collagenolytic activity has been emphasized by the recent crystallographic studies of the catalytic domain of MMP-8 (neutrophil collagenase) by Bode and colleagues (Bode et al., 1994; Reinemer et al., 1994). Their studies demonstrated structural stability by forming a salt bridge between the ammonium group of the N-terminal Phe\textsuperscript{37} (corresponding to Phe\textsuperscript{38} in MMP-1) and the side-chain carboxylate group of Asp\textsuperscript{32a} (Reinemer et al., 1994). Without Phe\textsuperscript{38}, the N-terminal hexapeptide Met\textsuperscript{36}-Leu-Thr-Pro-Gly-Asn\textsuperscript{38} was disordered (Bode et al., 1994). It is postulated that a small structural (motional) difference may lead to different stabilization of the active site and of the transition state, or the mobile N-terminal segment may interfere with substrate binding to the active site (Reinemer et al., 1994).

MMP-1 generated by RMCP II has either one extra residue (Gln\textsuperscript{80}) or lacks Phe\textsuperscript{38}. Such molecules are unlikely to form a stable N-terminal salt bridge with the carboxylate group of Asp\textsuperscript{32a}. Once these molecules are generated by RMCP II cleavage, MMP-3 can no longer participate in MMP-1 activation, as MMP-3 requires a stretch of at least four amino acids on the N-terminal side of the scissile bond for effective hydrolysis (Niedzwiecki et al., 1992).

To date no proteinases have been shown to activate purified proMMP-2, although in most cases it becomes degraded (Okada et al., 1990). The present study adds two more enzymes to this list, namely RMCP-I and II. Keskii-Oja et al. (1992) reported the processing of the 72 kDa proMMP-2 to a 62 kDa by urokinase, but no gelatinolytic activity was detected with the 62 kDa species unless it was treated with SDS. The inability of many endopeptidases to activate proMMP-2 may be explained by the unique sequence of the propeptide which corresponds to the ‘bait’ regions of the other proMMPs (Nagase et al., 1991). However, studies by Brown et al. (1990) and Overall and Sodek (1990) demonstrated that phorbol myristate acetate- or concanavalin A-treated fibroblasts and some tumour-cell lines express a cell surface-bound ‘activator’ of proMMP-2. This may be a key endogenous activator for proMMP-2, but the biochemical properties are not clear and have yet to be characterized.

Variation in proteinase content is a major feature of mast-cell heterogeneity in rat and man (Irani et al., 1986; Newlands et al., 1987). In the rat RMCP I is found in ‘connective tissue-type’ mast cells of skin, lung and muscle, whereas RMCP II is located mainly in the mucosal-type mast cells of the gut and airways (Gibson and Miller, 1986). In humans, trypatase is the dominant enzyme of most mast cells found in mucosal tissue of the gut, lung and airway, but mast cells containing both tryptase and the chymotrypsin-like enzymes (chymase) are found in all tissues, especially in skin and intestinal submucosa (Irani et al., 1986; Weidner and Austen, 1993). Together with the recent realization that mast cells may express a variety of cytokines, including tumour necrosis factor α and interleukin 1 (see Gordon et al., 1990; Galli et al., 1991), it seems plausible that mast cells may contribute to the local regulation of collagen and matrix metabolism. Such a view was expressed by Birkedal-Hansen et al. (1976) on the basis of procollagenase activation by mast-cell proteinases. More recent evidence in support of this theory includes the observation of mast-cell activation/degranulation in association with areas of connective-tissue lysis, such as cartilage erosion sites in rheumatoid arthritis (Bromley et al., 1984) and at the tumour periphery of rat mammary adenocarcinoma and other cancers (Dabbous et al., 1986, 1991). The soluble products released by mast cells have been shown to stimulate the production of proMMP-1 by fibroblasts and tumour cells in vitro (Yoffe et al., 1984; Dabbous et al., 1986), and to stimulate monocyte-macrophages to produce increased amounts of interleukin 1, a cytokine known to stimulate MMP production by connective-tissue cells (Yoffe et al., 1985). Thus it would appear that mast-cell activation in vitro may contribute to localized matrix degradation, both by the stimulation of MMP production by resident connective-tissue cells and by the subsequent activation of released precursors by mast-cell proteinases.

RMCP I and RMCP II are very similar in amino acid sequence, three-dimensional structure and specificity towards synthetic substrates (Le Trong et al., 1987; Woodbury et al., 1989). However, despite similarities with chymotrypsin and cathepsin G, the physiological roles of the two RMCPs remain uncertain, although it has been assumed they probably have similar functions (Woodbury et al., 1989). The report that RMCP I remains associated with secreted granule heparin whereas RMCP II freely dissociates from released granules (Schwartz et al., 1981) suggests a different extracellular distribution for the two enzymes following mast-cell activation.

Our studies have shown that in vitro both RMCP I and II can activate proMMP-3, only RMCP II was found to activate proMMP-1, and neither enzyme was capable of activating proMMP-2. These different actions on specific proMMPs support the concept that mast-cell subtypes with different proteinase compositions may assume tissue-specific regulatory roles for MMP-mediated degradation of matrix components. However, the relatively slow activation of proMMP-1 by RMCP II, and the reduced collagenolytic activity resulting from the unique cleavage site, may provide an active collagenase species with specific properties for matrix degradation in vivo. As yet it remains to be determined which active forms of MMP-1 are generated in vivo, this probably being related to the cellular composition and type of matrix remodelling in various tissue locations.

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