# Dog Mastocytoma Cells Secrete a 92-kD Gelatinase Activated Extracellularly by Mast Cell Chymase

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## Abstract

**Gelatinolytic metalloproteinases implicated in connective tissue remodeling and tumor invasion are secreted from several types of cells in the form of inactive zymogens. In this report, characterization of gelatinase activity secreted by the BR line of dog mastocytoma cells reveals a phorbol-inducible, ~ 92-kD, $Ca^{2+}$- and $Zn^{2+}$-dependent proenzyme cleaved over time to smaller, active forms. Incubation of cells with the general serine protease inhibitor, PMSF, prevented proenzyme cleavage and permitted its purification free of activation products. The $NH_2$-terminal 13 amino acids of the purified mastocytoma progelatinase are 50–67% identical to those of human, mouse, and rabbit 92-kD progelatinase (gelatinase B; matrix metalloproteinase-9). Degranulation of mastocytoma cells using ionophore A23187 greatly accelerated proenzyme cleavage, suggesting that a serine protease present in secretory granules hydrolyzed the progelatinase to active fragments. To identify the activating protease, cells were coincubated with ionophore and a panel of selective serine protease inhibitors. Soybean trypsin inhibitor and succinyl-L-Ala-Ala-Pro-Phe-chloromethylketone, which inhibit mast cell chymase, prevented progelatinase activation. Inhibitors of tryptase and dog mast cell protease (dMCP)-3, i.e., aprotinin or bis(5-amidino-2-benzimidazolyl) methane (BABIM), did not. In further experiments using highly purified enzymes, mastocytoma cell chymase activated 92-kD progelatinase in the absence of other enzymes or cofactors; tryptase and dMCP-3, however, had no effect. These data demonstrate that dog mastocytoma cells secrete a metalloproteinase related to progelatinase B that is directly activated outside of the cell by exocytosed chymase, and provide the first demonstration of a cell that activates a matrix metalloproteinase it secretes by cosecreting an activating enzyme. In mastocytomas, this pathway may facilitate tumor invasion of surrounding tissues, and in normal mast cells, it could play a role in tissue remodeling and repair. (*J. Clin. Invest.* 1996. 97:1589–1596.) Key words: matrix metalloproteinase • serine protease • mast cell • degranulation • enzyme activation**

## Introduction

Activators of matrix metalloproteinase (MMP)[1] proenzymes disrupt an interaction between a cysteine residue in a conserved propeptide sequence (PRCGVPD) and $Zn^{2+}$ in the active site. Ensuing autocatalytic cleavage of $NH_2$- and COOH-terminal sequences generates truncated, enzymatically active products (1). Once activated, MMPs collectively hydrolyze a broad range of matrix proteins, including collagens and elastin. Although compounds such as organomercurial agents, detergents, and other chaotropes initiate MMP activation in vitro (2), it is likely that proteases cleave the propeptide at specific sites to initiate zymogen activation in vivo. For example, plasmin activates three MMPs, including collagenase (MMP-1), stromelysin (MMP-3), and 92-kD gelatinase (MMP-9, gelatinase B) (3, 4). Stromelysin, the first MMP found to activate other MMPs, activates both collagenase and 92-kD gelatinase (4, 5). Since both plasmin and stromelysin themselves must be activated from their proforms, they may function as intermediaries in pathways initiated by other enzymes already secreted in active form.

Tryptase and chymase, which, respectively, are trypsin- and chymotrypsin-like mast cell serine proteases, can activate various MMPs either directly or indirectly in vitro. Mast cells store active tryptase and chymase together with histamine and heparin in secretory granules and release them upon IgE-mediated activation (6). While purified tryptase and chymase each activate stromelysin directly (7, 8), they activate collagenase by different mechanisms. Tryptase can activate collagenase indirectly via activation of urokinase, which then activates collagenase-activating plasmin (9), or by first activating stromelysin (7). In contrast, chymase can activate procollagenase directly by cleaving its propeptide (10). Thus, the products of activated mast cells provide alternative pathways for activating MMPs.

Mast cell hyperplasia and elevated extracellular levels of granule-associated mediators have implicated activated mast cells in a variety of disorders linked to deranged extracellular matrix destruction, turnover, and deposition. Biopsy specimens from patients with idiopathic pulmonary fibrosis demonstrated increased numbers of mast cells distributed in thickened and fibrotic alveolar septa (11). Analysis of biopsy tissue from a broader range of fibrotic lung disorders demonstrated a correlation between mast cell hyperplasia and the degree of fibrosis (12). Morphologic inspection of mast cell secretory granules also revealed diminished numbers and disorganized contents of granules, leading to the hypothesis that parenchy-

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1. *Abbreviations used in this paper:* AAPF-CK, Ala-Ala-Pro-Phe-chloromethylketone; APMA, aminophenylmercuric acetate; BABIM, bis(5-amidino-2-benzimidazolyl) methane; dMCP-3, dog mast cell protease-3; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase–associated lipocalin; SBTI, soybean trypsin inhibitor; TIMP, tissue inhibitor of metalloproteinase; TPA, 12-0-tetradecanoylphorbol-13-acetate.

mal fibrosis results from a chronic process of partial degranulation (11). Additional data have demonstrated that activated mast cells do in fact release stored mediators during tissue remodeling. For example, bronchoalveolar lavage fluid of scleroderma patients with radiographic evidence of fibrosing alveolitis revealed elevated levels of histamine and tryptase in addition to increased numbers of mast cells (13); and inflammatory synovial fluid from patients with osteoarthritis and rheumatoid arthritis demonstrated similar elevations of mast cell mediators (14).

We report here that BR mastocytoma cells, which are rich in mast cell tryptase, dog mast cell protease (dMCP)-3, and chymase (15–17), secrete a 92-kD gelatinase B–like MMP that is directly cleaved to active products by exocytosed chymase. These findings may reveal a potentially important pathway of MMP activation, linking products secreted by mast cells to destruction and turnover of extracellular matrix.

## Methods

*Materials.* Aminophenylmercuric acetate (APMA), aprotinin, casein, collagen (rat tail type I), DEAE cellulose, DMSO, gelatin agarose, In Vitro Toxicology LDH Assay Kit, ionophore A23187, lentil lectin Sepharose, α-methyl mannoside, 1,10-phenanthroline, PMSF, polyoxyethylene 23-lauryl ether (Brij 35), soybean trypsin inhibitor (SBTI), Triton X-100, 12-0-tetradecanoylphorbol-13-acetate (TPA), and porcine skin gelatin were obtained from Sigma Chemical Co. (St. Louis, MO). YM-10 filtration membranes were obtained from Amicon, Inc. (Beverly, MA). Succinyl-L-Ala-Ala-Pro-Phe-chloromethylketone (AAPF-CK) was obtained from Bachem Bioscience (King of Prussia, PA). *k*-Elastin was purchased from Elastin Products Co. (Owensville, MO). [$^3$H]Acetic anhydride was obtained from DuPont NEN Research Products (Boston, MA). Bis(5-amidino-2-benzimidazolyl) methane (BABIM) was kindly provided by Dr. Richard Tidwell (University of North Carolina, Chapel Hill, NC). Mast cell tryptase (16), chymase (15), and dMCP-3 (17) were purified from dog mastocytoma cells as described.

*Cell culture.* BR dog mastocytoma cells were maintained in continuous suspension culture in DME-H16 medium supplemented with 2% bovine calf serum at a final concentration of $1 \times 10^6$ cells/ml and incubated at 37°C in humidified 5% $CO_2$ and 95% air. The cells were harvested by centrifuging at 500 *g* for 5 min, washed three times in $Ca^{2+}$- and $Mg^{2+}$-free PBS, and then resuspended in serum-free DME-H16 to a final concentration of $6 \times 10^6$ cells/ml. Cells were stimulated with 25 ng/ml of TPA. The conditioned medium was harvested after 24 h and centrifuged at 500 *g* for 5 min to remove cells and debris; decanted supernatant was stored at −20°C.

*Zymography.* Gelatin substrate gels were prepared by incorporating gelatin (1 mg/ml) into 10% polyacrylamide gels containing 0.4% SDS. After electrophoresis under nonreducing conditions, the gels were washed twice for 30 min in 2.5% Triton X-100 (vol/vol) and incubated for 18 h at 37°C in a $Ca^{2+}$assay buffer consisting of 200 mM NaCl and 10 mM $CaCl_2$ in 40 mM Tris-HCl (pH 7.5). Gels were stained with Coomassie blue for 10 min and destained in 10% acetic acid/50% methanol. Clear zones of lysis against a blue background indicated gelatinase activity (18). Substrate zymography using casein or elastin was performed in a similar manner by incorporating casein or *k*-elastin (1.2 mg/ml) into 10% SDS-polyacrylamide gels.

To explore the $Ca^{2+}$ and $Zn^{2+}$ dependence of these metalloenzymes, substrate gels were incubated overnight in the presence of either 20 mM EDTA or 2 mM 1,10-phenanthroline. Purified dog chymase, a gelatin-degrading serine protease that is neither $Ca^{2+}$- nor $Zn^{2+}$-dependent, was assayed in separate lanes of the gel as a control. 2 mM 1,10-phenanthroline was added to $Ca^{2+}$ assay buffer, while 20 mM EDTA was added to $Ca^{2+}$-free assay buffer. 2 mM PMSF was also added to $Ca^{2+}$ assay buffer as a control.

*Degranulation.* Mastocytoma cells were harvested as described above and resuspended in serum-free DME-H16 medium to a final concentration of $25 \times 10^6$ cells/ml. Cells were then incubated alone at 37°C for 1 h with 2 μM A23187 alone, or with ionophore and 1 mM PMSF. Aliquots were removed at time 0 as a control and at specified intervals during the incubation period, and then centrifuged to remove cells. Supernatants were kept at −20°C before analysis by gelatin zymography. LDH cytotoxicity studies using the In Vitro Toxicology Assay Kit were performed on the supernatants to determine the amount of LDH released compared with total lysis of a control cell pellet as a measure of ionophore A23187–induced cell death.

*Purification.* Crude medium conditioned by mastocytoma cells was brought to a final concentration of 0.5 M NaCl by addition of NaCl and then subjected to batch chromatography on DEAE cellulose. The flow-through fractions containing the unbound enzyme were loaded directly onto an open gelatin agarose column ($1.5 \times 5$ cm) previously equilibrated in gelatin column buffer (50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl, 5 mM $CaCl_2$, 0.02% $NaN_3$, and 0.05% Brij 35). The bound enzyme was eluted with 10% DMSO (vol/vol) in the same buffer containing 1 M NaCl. The eluate was concentrated using a YM-10 filtration membrane and dialyzed against gelatin column buffer to remove the DMSO. The dialysate was loaded onto a lentil lectin Sepharose column ($1 \times 3$ cm). Bound enzyme was eluted with gelatin column buffer containing 0.5 M α-methyl mannoside. The eluate was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 10 mM $CaCl_2$. Gelatinase activity in eluate fractions was assayed by gelatin zymography. Purity of enzyme preparations was assessed on SDS-polyacrylamide gels stained with Coomassie blue.

*$NH_2$-terminal sequencing.* Amino acid sequence determination was performed by the Biomolecular Resource Center at the University of California, San Francisco. A 3-μg sample of the purified 92-kD gelatinase proenzyme was electrophoresed on a 12% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane stained with Coomassie blue. Bands at 92 and 22 kD were cut from the membrane and subjected individually to Edman degradation using a 470A gas-phase sequencer (Applied Biosystems, Foster City, CA) with an on-line 120A PTH analyzer (Applied Biosystems). Protein sequence database searches were carried out using GeneWorks software (Intelligenetics, Mountain View, CA).

*Inhibition of serine proteases.* Serine protease activity in culture medium was inhibited by adding PMSF at a final concentration of 1 mM. Mastocytoma cells were then incubated for 24 h alone or in the presence of TPA and/or PMSF. To deduce the serine protease(s) responsible for activation of gelatinase in culture, mastocytoma cells were incubated with a panel of selective inhibitors, including AAPF-CK, aprotinin, BABIM, SBTI, and PMSF. Harvested mastocytoma cells were resuspended in serum-free DME-H16 at a concentration of $25 \times 10^6$ cells/ml and incubated with individual inhibitors for 30 min at 37°C; an aliquot of cells incubated without inhibitors served as a control. After centrifugation to remove cells and debris, supernatants derived from these inhibitor incubations were assayed by gelatin zymography.

*Activation of purified gelatinase.* Tryptase, chymase, and dMCP-3 were purified from dog mastocytoma cells as described previously (15–17). A range of concentrations of the serine proteases were incubated with purified gelatinase at 37°C for 1 h; purified gelatinase was also incubated with 1–2 mM APMA as a control. The reactions were terminated by adding 1 mM PMSF and placing incubation solutions onto ice. Samples were analyzed by gelatin zymography.

*[$^3$H]Gelatin degradation.* 50 mg of rat tail collagen type I in 10 mM boric acid (pH 9.0) and 200 mM $CaCl_2$ buffer (1 mg/ml) were reductively acetylated with 5.0 mCi of [$^3$H]acetic anhydride to a specific activity of $2.0 \times 10^6$ cpm/mg (19). $^3$H-Labeled collagen was diluted with unlabeled collagen and dialyzed against 25 mM Tris-HCl (pH 7.6) buffer containing 1 mM $CaCl_2$ to yield a final specific activity of 150,000 cpm/mg of protein. The [$^3$H]collagen was denatured to [$^3$H]gelatin by boiling for 5 min immediately before use in assays. Pu-

rified samples of mastocytoma progelatinase were incubated with different concentrations of chymase at 37°C; aliquots were removed at various intervals and the reactions were terminated by addition of a range of concentrations of SBTI. The gelatinase samples were diluted in 25 mM Tris-HCl (pH 7.6) with 5 mM $CaCl_2$, 150 mM NaCl, 0.02% $NaN_3$, and 2 mg/ml ovalbumin, and then added to an equal volume of [$^3$H]gelatin. After incubation at 37°C for different time periods, the reactions were stopped by the addition of an equal volume of 50% trichloroacetic acid. Aliquots of supernatants were analyzed by liquid scintillation spectrometry to detect acid-solubilized gelatin peptides (20).

## Results

*Gelatinase expression.* As revealed by the gelatin zymogram shown in Fig. 1, mastocytoma cells secreted several size forms of gelatinolytic enzyme activity, which was constitutive as well as TPA-inducible. Serum-free medium conditioned by these cells for 24 h, examined under nonreducing conditions, contained major bands of activity at ~ 80 and 92 kD as well as bands in the range of 150–200 kD (which disappear under reducing conditions and may represent disulfide-linked dimers, as discussed below). Addition of TPA to cultured cells greatly increased levels of gelatinolytic activity associated with the major bands. Incubation of cells with 2 mM PMSF (a class-specific inhibitor of most serine proteases) in either the presence or absence of TPA eliminated most of the band (or bands) in the 80-kD region and reduced the intensity and multiplicity of the high molecular mass bands, indicating that generation of some of the bands depends in some fashion on serine proteases. However, as illustrated in the zymograms shown in Fig. 2, none of the bands of conditioned medium gelatinolytic activity were diminished by incubation of the gels themselves with PMSF, suggesting that serine proteases were not directly responsible for in situ degradation of gelatin in the gels. As a positive serine protease control, purified dog chymase was added to adjacent lanes, yielding a broad gelatinolytic band of ~ 30 kD, which virtually disappeared in gels incubated with PMSF, establishing that PMSF incubated with the gels in this manner can inhibit serine protease activity if present. On the other hand, 1,10-phenanthroline and EDTA (chelators of $Zn^{2+}$ and $Ca^{2+}$, respectively) ablated gelatinolytic activity of mastocytoma cell conditioned medium, establishing the dependence of the observed activity on these divalent cations and the likely identity of the gelatinases as MMPs. As expected, chelators did not affect the gelatinolytic activity of chymase. In addition to hydrolyzing gelatin, the various size forms of gelatinolytic metalloproteinase activity in mastocytoma cell conditioned medium cleaved casein and elastin in separate zymograms (not shown). Thus, zymography of medium conditioned by mastocytoma cells reveals multiple molecular forms of phorbol-inducible gelatinolytic metalloproteinase, which also cleaves casein and elastin. When medium was conditioned in the presence of PMSF, fewer bands were observed (with an ~ 92-kD band predominating), suggesting possible involvement of a serine protease in processing secreted metallogelatinases to alternative forms.

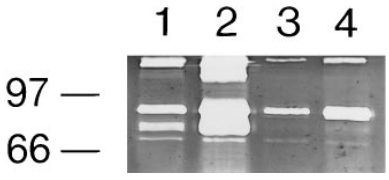


*Figure 1.* Effect of phorbol and PMSF on mastocytoma gelatinase expression. Dog BR mastocytoma cells were incubated with medium alone (lane *1*), medium plus 25 ng/ml TPA (lane *2*), medium plus 1 mM PMSF (lane *3*), or medium plus the combination of 25 ng/ml TPA and 1 mM PMSF (lane *4*). After 24 h, medium was collected and subjected to electrophoresis under nonreducing conditions in gelatin-containing 10% SDS-polyacrylamide gels. After incubation at 37°C for 18 h, gels were stained with Coomassie blue and destained with 10% acetic acid/50% methanol. TPA increased the intensity of multiple bands. PMSF decreased the number of bands both in the absence and presence of TPA. The faint ~ 72-kD band represents an as yet uncharacterized gelatinolytic protease. The size (in kilodaltons) and elution position of marker proteins are indicated to the left of the gel.

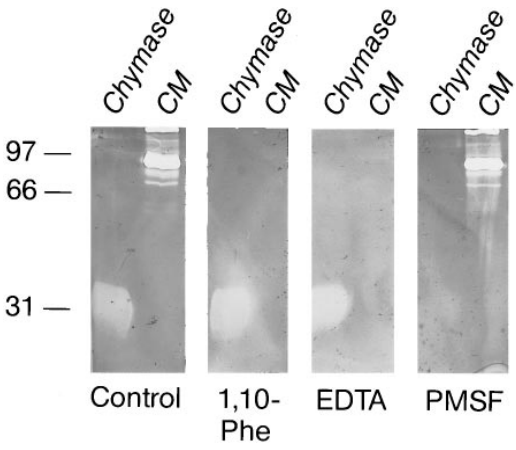


*Figure 2.* Effect of metal chelators and PMSF on gelatinase activity. Samples of mastocytoma cell conditioned medium (*CM*) and pure dog chymase (20 nM), a matrix protein–degrading serine protease used as a control, were subjected to gelatin zymography under nonreducing conditions. The gels were incubated overnight at 37°C in control $Ca^{2+}$ buffer alone (*Control*), with 2 mM 1,10-phenanthroline (*1,10-Phe*), with 20 mM EDTA in $Ca^{2+}$-free buffer (*EDTA*), and with 2 mM PMSF (*PMSF*). Note the activity of chymase at ~ 30 kD and conditioned medium predominantly at ~ 92 kD. 1,10-phenanthroline and EDTA block gelatinase activity in CM without affecting the gelatinase activity of chymase, while PMSF, a general serine protease inhibitor, blocks the latter without affecting the former.

*Gelatinase activation.* Because the major serine proteases of mast cells are stored in secretory granules, the potential role of mastocytoma cell granule-associated serine proteases in gelatinase activation was explored in further experiments. Medium conditioned by unstimulated cells demonstrates a principal band at ~ 92 kD; incubation beyond 8 h results in the appearance of an additional ~ 80-kD band, or a pair of closely spaced bands (data not shown). The zymograms depicted in Fig. 3 show the impact of mastocytoma cell degranulation on the electrophoretic pattern and order of appearance of gelatinolytic bands. In medium conditioned by unstimulated cells (i.e., not exposed to ionophore A23187), the ~ 92-kD band appeared within 5 min of addition of medium. By contrast, in medium conditioned by cells stimulated with 2 μM ionophore, the ~ 80-kD activity appeared much more quickly (within 5 min), progressively increasing in intensity over the next hour. Of the pair of bands in the 150–200-kD range, the topmost band (which is probably a homodimer of 92-kD subunits because it disappears in gels run under reducing conditions) predominates at time 0 in the medium conditioned by cells exposed to ionophore A21287 and at all time points in both the control conditioned medium and in the medium exposed to both iono-

phore and PMSF. However, in the A23187 alone lanes the topmost band virtually vanishes by 60 min (concomitant with the loss of the 92-kD band) and the lower of the two dominant high molecular mass bands progressively increases (concomitant with the increase in the ~ 80-kD band). This is further evidence that the top pair of bands are dimers made up of 92- and 80-kD enzymes, respectively; it also suggests that the fraction of gelatinase in the dimeric form is as susceptible to proteolytic downsizing as the monomeric form. Concurrent addition of 2 mM PMSF to ionophore-stimulated cells largely prevented the appearance of the 80-kD band, as shown in Fig. 3. Collectively, these results suggest that a granule-associated serine protease released by ionophore A23187 cleaves a monomeric or dimeric ~ 92-kD gelatinase to an active, smaller form with a subunit of ~ 80 kD. Additionally, the zymograms in Fig. 3 (and also in Fig. 4) consistently manifest an overall increase in the intensity of parent gelatinolytic bands after exposure of cells to ionophore, suggesting that in addition to the gelatinase activity that is secreted constitutively by unstimulated cells, an additional fraction may be subject to acute, regulated release under the influence of ionophore.

*Selective inhibition.* To identify the PMSF-inhibited protease responsible for the cleavage of the 92-kD gelatinase to its ~ 80-kD form, medium was collected from ionophore-stimulated cells coincubated with a panel of more selective protease inhibitors, as shown in Fig. 4. Medium conditioned by cells without ionophore A23187 revealed a major band of gelatinase activity at 92 kD; medium conditioned by cells incubated with 2 μM ionophore revealed new major activity at ~ 80 kD and more intense bands at 92 kD. As noted above, the increased intensity of the 92-kD band in ionophore-exposed cells may reflect release of a preformed pool of metallogelatinase activity. Coincubation of ionophore-treated cells with PMSF (but not coincubation with aprotinin or BABIM) blocked the appearance of the ~ 80-kD band, suggesting that neither tryptase nor dMCP-3 hydrolyzes the 92-kD gelatinase (16, 17, 21); however, SBTI and AAPF-CK, which inhibit chymase but not tryptase or dMCP-3 (15–17, 21), prevented appearance of the lower molecular mass band. Cells incubated with inhibitors released 5–8% of total LDH into the supernatant; this level of LDH release was the same as that obtained from cells incubated with ionophore alone or together with inhibitors, or with neither ionophore nor inhibitors. These experiments implicate mastocytoma cell chymase in the cleavage of the 92-kD metallogelatinase to active, smaller forms.

*Gelatinase purification.* To allow further dissection of the phenomena identified in the above studies, mastocytoma gelatinase was purified free of other enzymes. Cultured cells were incubated with TPA to maximize gelatinase production and with PMSF to prevent serine protease–mediated activation and allow isolation of the proenzyme free of cleavage products. As shown in Fig. 5, gelatinase purified from medium conditioned by cells cultured under these conditions was analyzed by nonreducing zymography and standard SDS-PAGE, which demonstrated a predominant band at 92 kD plus less prominent high molecular mass bands. The latter bands disappeared under reducing conditions, as shown in lane *3*. In addition, an ~ 22-kD band was observed on SDS-PAGE gels under nonreducing and reducing conditions; it did not demonstrate gelatinolytic activity by zymography. $NH_2$-terminal sequencing revealed this protein to be lentil lectin, which apparently is a column contaminant copurified due to gelatinase affinity. No

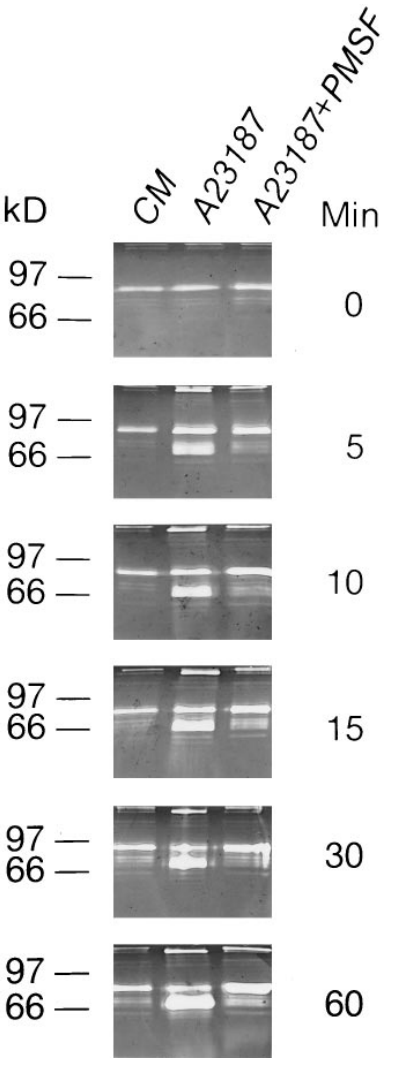


*Figure 3.* Effect of degranulation by ionophore A23187 on gelatinase activity. Aliquots of supernatants from mastocytoma cells incubated with medium alone (*CM*), with medium plus 2 μM A23187, or medium plus a combination of 2 μM A23187 and 2 mM PMSF were sampled at the indicated intervals and then assayed by gelatin zymography. Note the prompt appearance of new bands upon degranulation by ionophore and the change in intensity of bands over time, which is especially striking for activity in the ~ 80-kD range. Activity of this band becomes progressively more intense over time. PMSF largely blocks this effect, yielding a profile of bands similar to that generated by conditioned medium alone.

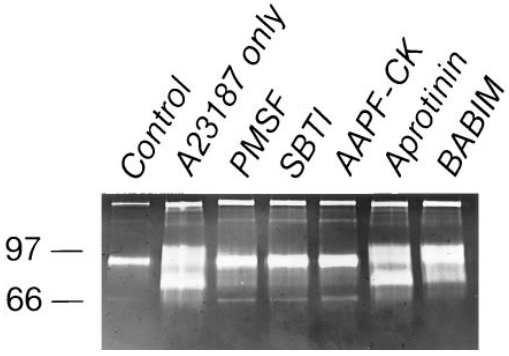


*Figure 4.* Effect of selective serine protease inhibitors on ionophore-induced progelatinase activation. Aliquots of medium conditioned by cells incubated with medium alone (*Control*), with medium plus 2 μM ionophore A23187 (*A23187 only*), and medium with a combination of A23187 and a protease inhibitor: 2 mM PMSF, 12.5 nM SBTI, 0.5 mM AAPF-CK, 15 nM aprotinin, or 0.5 mM BABIM. Note that the control medium yields activity predominantly at ~ 92 kD. A23187 results in appearance of a major ~ 80-kD band and a more intense band at 92 kD. Inhibitors of chymase (PMSF, SBTI, and AAPF) block appearance of the ~ 80-kD band, while inhibitors of tryptase and dMCP-3 (aprotinin and BABIM) do not.

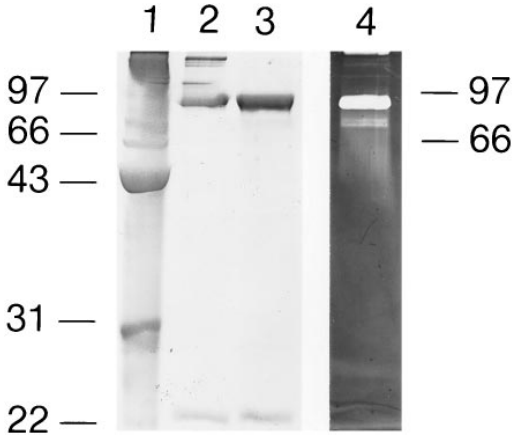


*Figure 5.* Purification of mastocytoma gelatinase. Crude medium conditioned by BR mastocytoma cells in the presence of phorbol and PMSF was purified by DEAE cellulose ion-exchange, gelatin affinity, and lentil lectin chromatography. Purity was assessed by SDS-PAGE and Coomassie blue staining (lanes *1–3*) and gelatin zymography (lane *4*). Lane *1* contains protein from crude unpurified medium. Lanes *2* and *3* contain purified gelatinase exposed to nonreducing and reducing conditions, respectively. Analysis of the purified protein by gelatin zymography (lane *4*) revealed activity at ~ 92 kD and minor activation products, without evidence of other gelatin-degrading proteases. The ~ 22-kD band was a lentil lectin protein contaminant without gelatinolytic activity.

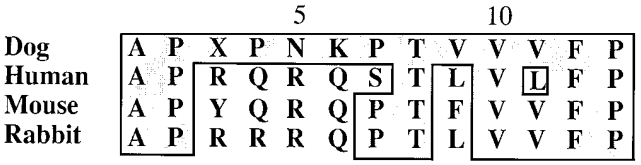


*Figure 6.* Comparison of gelatinase $NH_2$-terminal amino acid sequences. After SDS-PAGE and transfer of the dog 92-kD progelatinase to a polyvinylidene difluoride membrane, the $NH_2$-terminal amino acid sequence was determined by automated Edman degradation. The dog mastocytoma gelatinase sequence was compared with $NH_2$-terminal regions of human and rabbit 92-kD progelatinases and mouse 105-kD progelatinase. Regions of identity are boxed and shaded.

proteins similar to the three known tissue inhibitors of metalloproteinases (TIMP-1, 28.5 kD; TIMP-2 or TIMP-3, both 21 kD) (22–24) or to neutrophil gelatinase–associated lipocalin (NGAL, 25 kD) (25), were identified. In summary, the culture and purification strategy yielded a 92-kD protein free of contamination by inhibitors and other gelatinases.

*$NH_2$-terminal amino acid analysis.* Edman degradation of 92-kD gelatinase purified from BR mastocytoma cell culture supernatants yielded the following sequence: APXPNKPTV-VVFP. No residue was assigned in cycle 3 due to insufficient discrimination of chromatographic peak amplitudes from the prior and subsequent cycles. The identification of Pro in cycle 4 was also accompanied by an increase in the Gly peak. Cycle 5 yielded an Asn peak, although a less intense His peak was also detectable. Fig. 6 compares this sequence with that of human and rabbit 92-kD progelatinases (26, 27) and mouse 105-kD progelatinase (the murine homologue of 92-kD gelatinase/gelatinase B/MMP-9) (28). 6 of the 12 identified residues are shared among the four species, with variability between residues three through seven. The dog sequence is 67% identical to the rabbit and mouse sequences and 50% identical to the human sequence, but is only 23% identical to the corresponding $NH_2$ terminus of human 72-kD progelatinase (29). These data buttress the zymographic evidence that the mastocytoma progelatinase is a member of the 92-kD gelatinase/gelatinase B/MMP-9 family of MMPs.

*Cleavage of purified gelatinase by chymase.* Purified mastocytoma progelatinase was reconstituted with purified mastocytoma chymase to determine whether involvement of the latter enzyme in the cleavage of progelatinase to active, smaller forms is a direct effect or one mediated by intermediary enzymes possibly present in crude conditioned medium. As shown in Fig. 7, purified gelatinase demonstrated activity predominantly at 92 kD, with additional higher molecular mass bands presumably representing gelatinase dimers as noted above. Activation by APMA, which is thought to stimulate autolytic cleavage in vitro by modifying the free cysteine liganded to the catalytic $Zn^{2+}$ (1, 4, 30), yielded active products at ~ 67 (faint), ~ 74, and ~ 83 kD. Incubation with a range of concentrations of chymase generated a similar pattern of gelatinase activation products (at ~ 67, ~ 74, and ~ 83 kD), with progressive disappearance of proenzyme activity with increasing chymase concentration along with progressively more intense activity at ~ 67 kD. The diminished activity at ~ 92 kD over the same interval suggests that the proenzyme is the parent of the activated products. Prolonged electrophoresis of these activation products generated by increasing chymase/progelatinase ratios, as seen in Fig. 7, improves separation of bands of chymase-stimulated activation products. Chymase alone yielded no gelatinolytic activity in this molecular mass range (refer also to Fig. 2). In contrast, incubation under the same conditions with tryptase (60 nM) and dMCP-3 (50 nM) had no effect on the zymographic profile of the purified mastocytoma progelatinase (not shown). Thus, these experiments suggest that chymase directly activates mastocytoma gelatinase. The size similarity of the bands resulting from incubation of mastocytoma progelatinase with APMA and chymase is consistent with a mechanism in which both agents stimulate autolytic cleavage by the gelatinase at similar or identical sites.

*Soluble activity of chymase-activated 92-kD gelatinase.* To determine whether chymase cleavage of 92-kD gelatinase yields products that are active in solution, gelatinolytic activity of the cleavage products was assessed by their ability to digest $^3$H-labeled gelatin. This is a potentially more biologically relevant test of chymase-mediated activation than zymography in which matrix metalloenzymes, including proforms, are activated artificially by SDS (2). The time course shown in Fig. 8 illustrates the activity of 92-kD gelatinase in the absence and presence of chymase. Gelatinase activity detected at time 0 reflects the presence of active forms resulting from purification and autoactivation during storage. In the absence of chymase, soluble gelatinase activity decreases during the incubation period. By contrast, incubation with chymase yields an approximately twofold increase in activity at 4 h, followed by a steeper rate of inactivation; by 8 h the activity is indistinguishable from gelatinase unexposed to chymase. Chymase itself demonstrates at each incubation interval low levels of gelatinolytic activity at no more than 5% of the initial gelatinase activity (data not shown). Thus, 92-kD progelatinase is activated by chymase to yield products which degrade gelatin in solution.

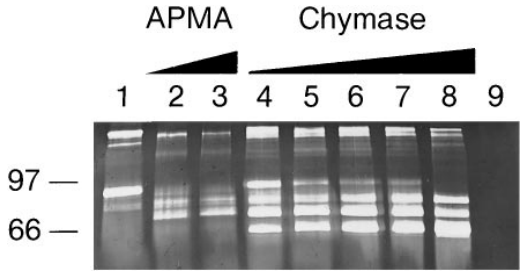


*Figure 7.* Effect of purified mastocytoma chymase on processing of purified mastocytoma progelatinase. Aliquots of purified gelatinase (1.5 nM) were incubated for 20 min at 37°C with APMA (1.0 or 2.0 mM, lanes *2* and *3*) or increasing concentrations of chymase (20, 40, 100, 200, and 400 nM, lanes *4–8*) and then subjected to gelatin zymography under nonreducing conditions. As an additional control, chymase alone (400 nM) was loaded onto lane *9*. The activity of progelatinase alone is shown in lane *1*. Progelatinase incubated with APMA yields products at ~ 92, 83, 74, and 67 kD. Progelatinase incubated with increasing concentrations of purified dog chymase yield products at 92, 83, 74, and 67 kD with progressive increase in intensity of the 67-kD band and disappearance of the 92-kD band. Chymase alone (400 nM) has no gelatinolytic activity between 66 and 97 kD. These results suggest that chymase directly activates mastocytoma gelatinase.

## Discussion

This work describes the purification and features of the major gelatinase secreted by dog mastocytoma cells and identifies mast cell chymase as the product of these cells responsible for degradation of the gelatinase proenzyme to active forms. Several key characteristics of the mastocytoma gelatinase suggest that it is a member of the 92-kD gelatinase/gelatinase B/MMP-9 family of MMPs. These features include electrophoretic mobility similar to that of a human 92-kD gelatinase (18), a tendency to oligomerize (18), secretion in a constitutive as well as phorbol-inducible manner, inhibition by chelators of $Ca^{2+}$ and $Zn^{2+}$, and an ability to degrade gelatin, casein, and elastin (26, 31). Like other MMPs, 92-kD gelatinases are secreted as inactive zymogens (32). Proteolysis of 8–10 kD of the $NH_2$-terminal domain activates the proenzyme and enables it to digest collagens type IV and V, gelatin, elastin, and fibronectin (18, 26, 31). While the organomercurial agent, APMA, and another MMP, stromelysin, can initiate progelatinase activation in vitro (1, 4, 30), physiologic activation mechanisms are unknown.

The data presented here show that dog mastocytoma 92-kD gelatinase, like similar enzymes secreted from cultured leukocytes, is secreted initially as an inactive zymogen. However, in contrast to other cultured cells expressing 92-kD gelatinase activity mastocytoma cells rapidly degrade the proenzyme to active derivatives. Nonetheless, in their constitutive secretion of the 92-kD progelatinase and in their ability to increase production in response to phorbol, the dog mastocytoma cells are similar to human alveolar macrophages and T lymphocytes (33, 34). The existence of phorbol-responsive secretion in these cells suggests that gelatinase gene expression is regulated by transcription factors AP-1 or AP-2, which control cell type–specific expression in a manner possibly unique (among MMPs) to 92-kD gelatinase (27, 35).

In its electrophoretic profile, the purified dog proenzyme is similar to that of human neutrophil 92-kD gelatinase (18). Like the human neutrophil proenzyme, dog progelatinase yields multiple forms of apparent molecular mass > 92 kD, including bands in the 200-kD range, when analyzed by SDS-PAGE or gelatin zymography under nonreducing conditions. These slow-migrating bands disappear in the presence of reducing agents, indicating that they are formed of smaller components linked by disulfide bonds. The major band in the ~ 200-kD range is likely to be a dimer composed of 92-kD subunits, as is the case of a similar band in preparations of human neutrophil gelatinase (18). In all preparations of mastocytoma gelatinase examined, the presumed dimer fraction is minor relative to the ~ 92-kD monomer fraction. Of particular interest is the increase in intensity of a band (migrating slightly faster than the suspected proenzyme dimer band and seen with careful scrutiny of Figs. 1, 3, and 4) concomitant with the build-up of the chymase-generated ~ 80-kD derivative of the 92-kD

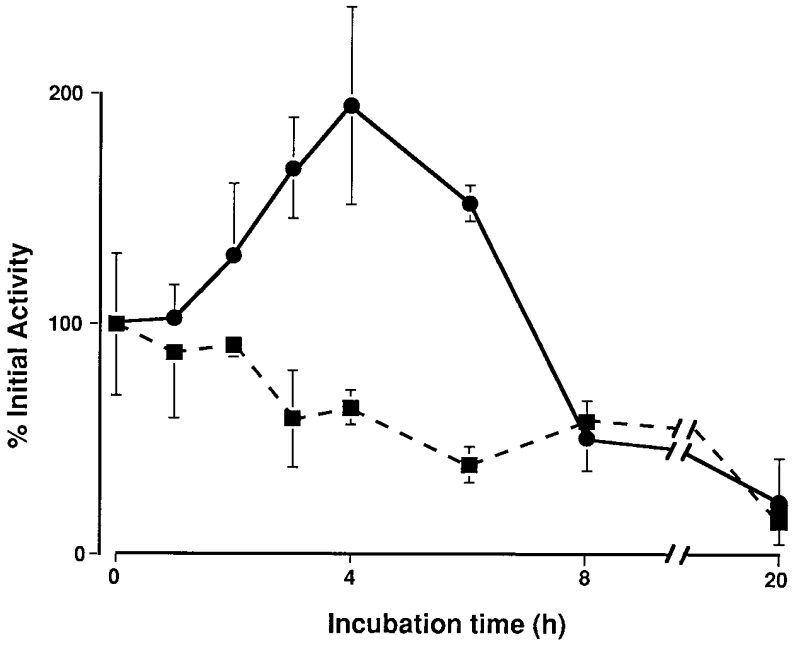


*Figure 8.* Gelatin degradation assay. Purified gelatinase (40 nM) in the absence or presence of purified mastocytoma chymase (16 nM) and chymase alone (16 nM) were incubated at 37°C for 20 h in 250 μl of 50 mM Tris-HCl (pH 7.6) buffer containing 10 mM $CaCl_2$. Aliquots were removed at the indicated time intervals and the reactions were stopped by the addition of SBTI (1.5 mM). Gelatinase activity in the removed aliquots was determined by incubation of samples with boiled [$^3$H]collagen for 2 h (in triplicate). Activity of gelatinase alone (*boxes*) decreased over the incubation period. The activity of chymase-exposed gelatinase (*circles*) increased compared with gelatinase alone, peaking at 4 h. The peak activity of chymase alone (not shown) was no greater than 5% of initial gelatinase activity at any of the sampling intervals and therefore made a negligible contribution to digestion of collagen in this assay.

proenzyme and concomitant with the disappearance of the uppermost (progelatinase dimer) band. The new high molecular mass band is likely to be a dimer of 80-kD gelatinase and is of interest because it may be active under physiological conditions and because its presence suggests that chymase may be able to cleave and activate mastocytoma progelatinase in dimeric as well as monomeric form. One or more of the still fainter bands that are especially evident in lane *2* of Fig. 5 may represent the progelatinase disulfide-linked to the dog homologue of 25–50-kD human NGAL (18, 25), a protein of unknown function. However, no unidentified protein in this size range is seen on reduced gels, perhaps because at best the NGAL-linked population of gelatinase is a very minor fraction of the total pool. SDS-PAGE analysis also failed to reveal the presence of any dog homologues of human TIMPs, suggesting that mastocytoma cells, like neutrophils, secrete a 92-kD gelatinase that is TIMP free (18).

Whereas crude supernatants of gelatinase-producing human cells usually reveal predominant activity only at 92 kD (i.e., the proenzyme), unpurified mastocytoma supernatants demonstrate additional activity at ~ 80 kD. Several lines of evidence suggest that this band represents a proenzyme cleavage product rather than a second proenzyme. Because MMPs resist serine protease inhibitors, ablation of activity at ~ 80 kD upon coincubation of cells with PMSF implicates serine proteases in the generation of the 80-kD band. The prompt appearance of a similar band in degranulation supernatants and its attenuation by PMSF suggested that proteases present in secretory granules are responsible for progelatinase activation. Coincubation of mastocytoma cells with inhibitors selective for the known mast cell serine proteases enabled identification of chymase as the progelatinase activator. Chymase activation of 92-kD gelatinase results in a burst of gelatinolytic activity followed by a rapid decline. Cleavage of the propeptide by chymase may accelerate conversion of the proform to active intermediates, yielding a transient accumulation of active products. Rapid autodegradation likely ensues favoring conversion of the active forms to inactive, truncated fragments.

Since serine proteases have little intragranular activity due to the unfavorable acidic milieu of the granule, it is unlikely that activation of progelatinase by chymase occurs within secretory granules (6). However, the increase in intensity of the ~ 92-kD bands upon stimulation of cells by ionophore, seen most clearly in Fig. 4, does suggest the possibility of intragranular storage of 92-kD progelatinase, as is the case in neutrophils (36). Release of stored proenzyme upon degranulation may maintain levels of the 92-kD form even as activation products accrue. This may explain why the ~ 92-kD proenzyme parent band in Fig. 7 diminishes in intensity in the presence of increasing concentrations of activating chymase, but remains almost unchanged in degranulation reactions depicted in Fig. 3, even as activation products accumulate. Indeed, the data further suggest that chymase and mastocytoma progelatinase may lie in separate intracellular compartments. If mastocytoma chymase and progelatinase were cosecreted from the same granule or compartment, one would expect to see activation of the 92-kD proenzyme secreted constitutively from mastocytoma cells in the absence of stimulation by ionophore. In fact, as seen in Fig. 3, there is very little activation, even over 60 min of conditioning, whereas one sees obvious activation even at the earliest time point (5 min) after stimulation with ionophore. These findings suggest that the secretory granules containing chymase and progelatinase are functionally and probably physically distinct and that progelatinase activation occurs outside of the cell. The dependence of the proposed proMMP activation mechanism on exocytosis of stored proteases differentiates it from two previously reported pathways studied in cells transfected with MMP plasmid cDNA. Intracellular activation of prostromelysin in tumor cells can be initiated by furin, a Golgi-associated serine protease (37). In addition, activation at the cell surface can also occur as illustrated by cleavage of 72-kD progelatinase by membrane-type MMP (38). The current work, which involves soluble secreted proteases, describes a pathway for extracellular activation.

The successful reconstitution of events observed in crude conditioned medium using chymase and progelatinase purified from mastocytoma cells essentially rules out involvement by other secreted mediators in the proposed mechanism. The electrophoretic homogeneity of the reduced, purified dog progelatinase sharply reduces the possibility of participation by any intermediary proteases, including stromelysin, which activates human 92-kD gelatinase in vitro (4). In this respect, the proposed mechanism resembles the direct activation of human procollagenase by human skin chymase, which hydrolyzes the $Leu^{83}$-$Thr^{84}$ bond of procollagenase (10); whether a similar hydrolysis site in dog 92-kD gelatinase is recognized by chymase remains to be determined. These results contrast with those of a previous report which demonstrated that chymase in dog mastocytoma cell extracts could not independently activate a 92-kD progelatinase purified from a human breast carcinoma cell line (8). In the latter work the use of an unpurified and uncharacterized dog chymase (potentially an isoform that differs from purified BR mastocytoma chymase) in potentially different enzyme/substrate ratios on a substrate isolated from a different species may account for the discrepancy.

Because binding of TIMP can affect proMMP processing, the absence of TIMP from purified dog gelatinase predicted that its activation may be similar to that of another TIMP-free proMMP, human neutrophil 92-kD gelatinase. APMA fully activates the TIMP-free neutrophil enzyme resulting in 83-, 74-, and 67-kD cleavage products. The addition of TIMP in vitro blocks proenzyme processing beyond the 83-kD form (18, 39). Like TIMP-free neutrophil gelatinase treated with APMA, TIMP-free dog 92-kD progelatinase incubated with chymase is pared down to the 67-kD form; the functional differences between these size forms of cleaved 92-kD gelatinases are not yet clear. It also remains to be determined whether absence of TIMP favors a rapid, initial activation of mastocytoma progelatinase and/or hastens its processing to inactive forms, thereby limiting its half-life.

Several features revealed by the dog mastocytoma model suggest that mast cells may contribute active MMPs to homeostatic and pathologic tissue remodeling in a controlled manner. In addition to constitutive secretion, phorbol-inducible 92-kD progelatinase expression implicates possible transcriptional regulation by factor AP1, which is important for selective tissue expression in vivo (27). Localization of gelatinase in a distinct class of secretory granules in human neutrophils (36) suggests that the enzyme may be packaged similarly in mast cells, permitting regulated release in response to appropriate triggers (perhaps the same triggers, such as ionophore, that release chymase). Dependence upon exocytosed chymase for activation may limit generation of active gelatinase by the proposed mechanism in additional ways. As only a minority of

human mast cells produce chymase, this pathway would be available only to a distinct subset distributed in well-defined anatomic sites (6). Degranulation also requires mast cell stimulation (6), thus further restricting progelatinase activation to environments with the appropriate IgE-dependent and independent conditions. Given that the inflammatory milieu is normally multicellular and rich in secreted proteases, inhibitors, and other mediators, the physiologic activation of 92-kD gelatinase released by mast cells is certain to be complex.

In summary, our results demonstrate that dog mastocytoma cells secrete a 92-kD gelatinase which is activated outside of the cell by chymase. Mastocytoma 92-kD gelatinase is the second MMP found to be directly cleaved and activatable by chymase. The novel demonstration of extracellular activation of 92-kD progelatinase by chymase suggests that cells can use secondary proteases to activate MMP proenzymes not only in the cytosol and on the cell membrane surface, but also in the immediate extracellular milieu. Furthermore, these results predict that chymase secreted by activated mast cells in tissues will cleave and activate latent 92-kD gelatinase secreted by other types of inflammatory and stromal cells.

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