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ARTICLES

Pericellular Substrates of Human Mast Cell Tryptase: 72,000 Dalton Gelatinase and Fibronectin

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Abstract Migrating cells degrade pericellular matrices and basement membranes. For these purposes cells produce a number of proteolytic enzymes. Mast cells produce two major proteinases, chymase and tryptase, whose physiological functions are poorly known. In the present study we have analyzed the ability of purified human mast cell tryptase to digest pericellular matrices of human fibroblasts. Isolated matrices of human fibroblasts and fibroblast conditioned medium were treated with tryptase, and alterations in the radiolabeled polypeptides were observed in autoradiograms of sodium dodecyl sulphate polyacrylamide gels. It was found that an M_r 72,000 protein was digested to an M_r 62,000 form by human mast cell tryptase while the plasminogen activator inhibitor, PAI-1, was not affected. Cleavage of the M_r 72,000 protein could be partially inhibited by known inhibitors of tryptase but not by aprotinin, soybean trypsin inhibitor, or EDTA. Fibroblastic cells secreted the M_r 72,000 protein into their medium and it bound to gelatin as shown by analysis of the medium by affinity chromatography over gelatin-Sepharose. The soluble form of the M_r 72,000 protein was also susceptible to cleavage by tryptase. Analysis using gelatin containing polyacrylamide gels showed that both the intact M_r 72,000 and the M_r 62,000 degraded form of the protein possess gelatinolytic activity after activation by sodium dodecyl sulphate. Immunoblotting analysis of the matrices revealed the cleavage of an immunoreactive protein of M_r 72,000 indicating that the protein is related to type IV collagenase. Further analysis of the pericellular matrices indicated that the protease sensitive extracellular matrix protein fibronectin was removed from the matrix by *tryptase in a* dose-dependent manner. Fibronectin was also susceptible to proteolytic degradation by tryptase. The data suggest a role for mast cell tryptase in the degradation of pericellular matrices.

Key words: gelatinase, type IV collagenase, fibronectin, tryptase, proenzyme activation, mast cell, basement membrane, pericellular matrix

Most normal and malignant cell lines of both fibroblastic and epithelial origin secrete and deposit into their pericellular matrices M_r 66,000–72,000 proteins [Keski-Oja and Todaro, 1980; Vartio and Vaheri, 1981].[1] Transforming growth factor-β (TGFβ) enhances both the secretion of the M_r 66,000 protein and its deposition to the pericellular matrix in cultured fibroblasts [Keski-Oja et al., 1986]. Vartio and Vaheri [1981] described a gelatin binding soluble protein of M_r 70,000 that was immunologically distinct from the other known gelatin-binding proteins of M_r 95,000 [Vartio et al., 1982] and 440,000 (fibronectin). Subsequently, the soluble M_r 70,000 protein was found to possess gelatinolytic activity and to be possibly related to gelatinase/type IV procollagenase on the basis of identical amino terminal sequences [Vartio and Baumann, 1989]. Our recent analyses have indicated that the matrix-associated M_r 66,000–72,000 protein and the gelatin binding soluble M_r 70,000 protein co-migrate in SDS-polyacrylamide gels. These proteins thus appear to be identical and the observed differences in their molecular weights are likely due to differences in the analysis conditions.

[1]The molecular weights of the native (M_r 72,000) and proteolytically processed (M_r 62,000) forms of the gelatinolytic enzyme dealt with in this article are based on their electrophoretic mobility in reduced SDS-PAGE. For the sake of clarity these molecular weights will be used also when referring to corresponding polypeptides in nonreduced gels, where the protein migrates as an M_r 66,000 protein [Vartio and Baumann, 1989; Overall et al., 1989, 1991]. This is justified also because the calculated molecular weight of the active type IV collagenase based on the amino acid sequence of the protein is 62,067 daltons while the precursor is 70,984 daltons [Huhtala et al., 1990].

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The M_r 72,000 (66,000) fibroblast matrix-associated protein is susceptible to cleavage by various proteinases including trypsin, thrombin, the plasminogen activator urokinase, and proteases from the conditioned medium of 8387 fibrosarcoma cells [Keski-Oja and Todaro, 1980; Keski-Oja et al., 1981, 1992; Keski-Oja and Vaheri, 1982]. Cleavage of the 72,000 dalton protein occurs also to some extent in vivo as seen in cell culture analyses [Keski-Oja et al., 1981; Vartio and Baumann, 1989]. Like intact M_r 72,000 protein the M_r 62,000 cleavage product is retained in the pericellular matrix, and in some cases the cleavage of the M_r 72,000 protein is associated with the release of fibronectin from the matrix [Keski-Oja and Todaro, 1980; Keski-Oja et al., 1981].

Tryptase is a neutral trypsin-like serine protease that is essentially confined to mast cell granules. Biologically active tryptase is a tetramer of M_r 120,000–145,000 consisting of four subunits of about M_r 35,000–37,000 each [Schwartz et al., 1981]. The active tetrameric form is stabilized with heparin and protein (e.g., albumin) [Schwartz and Bradford, 1986; Harvima et al., 1988a] or with high salt concentration [Harvima et al., 1988a,b]. Tryptase is not capable of degrading proteoglycans or type I collagen. However, it has been reported to activate latent rheumatoid synovial procollagenase [Gruber et al., 1988], although contradictory results have also been published [Johnson and Cawston, 1985]. The functions of the enzyme in vivo are not known.

In the present study we have analyzed the ability of human mast cell tryptase to digest isolated pericellular matrices and compared the effects to those of chymase. We found that tryptase is able to use as its substrate the M_r 72,000 protein present in human fibroblast extracellular matrices and conditioned medium to yield lower molecular weight (M_r 62,000) and slightly smaller forms. Analysis of the soluble forms of the protein by gelatin zymography (where SDS activates gelatinase activity) indicated that both the high and low molecular weight forms have gelatinolytic activity. Immunoblotting analysis of matrix-associated M_r 72,000 polypeptides using peptide antibodies against type IV collagenase indicated that the intensity of the M_r 72,000 band was decreased by tryptase. Recombinant M_r 72,000 type IV collagenase/gelatinase was also cleaved by tryptase as shown by gelatinolysis zymography. Pericellular matrices of adherent cells frequently contain two other major proteins, fibronectin [Hedman et al., 1979] and plasminogen activator inhibitor type-1, PAI-1 [Laiho et al., 1986a,b; see Laiho and Keski-Oja, 1989], which are susceptible to the action of a number of serine proteinases. We found that fibronectin is released from the matrix by tryptase and degraded to fragments of lower molecular weight. No reproducible effects on PAI-1 were noted. The results indicate that human mast cell tryptase has a number of physiological regulatory roles including direct degradation of pericellular matrices and possibly activation of latent type IV collagenolytic activity.

MATERIALS AND METHODS

Reagents

Porcine platelet derived TGFβ1 was purchased from R&D Systems (Minneapolis, MN). Aprotinin, heparin, SBTI (soybean trypsin inhibitor), PMSF (phenyl methyl sulphonyl fluoride), antipain, leupeptin, BSA (bovine serum albumin), and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO). Gelatin-Sepharose 4B was purchased from *Pharmacia* (Uppsala, Sweden). Human mast cell tryptase was purified from normal human skin as described in detail [Harvima et al., 1988a]. Rat chymase was a kind gift of Dr. Tapio Vartio (Department of Pathology, University of Helsinki, Finland) [Vartio et al., 1981]. Recombinant M_r 72,000 type IV collagenase/gelatinase produced in a baculovirus system was kindly provided by Drs. Ari Tuuttila and Karl Tryggvason (Department of Biochemistry, University of Oulu, Finland). Purified peptide antibody against amino acid residues 472–490 of M_r 72,000 type IV collagenase sequence as described [Höyhtyä et al., 1988] was used in immunoblotting. Polyclonal antibody against human plasma fibronectin was a gift of Dr. Antti Vaheri (Department of Virology, University of Helsinki).

Cell Cultures and Treatment With Growth Factors

Human embryonic lung fibroblasts (CCL-137, HEL 299) were cultivated in medium 199 containing 10% heat-inactivated fetal calf serum (GIBCO), 100 IU/ml penicillin, and 50 μg/ml streptomycin. The cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. Confluent cultures were washed with serum-free medium 199 and then incubated under serum-free conditions for 6–8 h. The medium was then replaced by fresh medium 199, TGFβ1 was added, and the cultures were incubated for an additional 18–24 h. All experiments were carried out under serum-free conditions.

Radioactive Labeling and Polypeptide Analyses

Confluent cultures of CCL-137 cells were labeled with [^{35}S]methionine (50 μCi/ml, > 1,000 Ci/mmol; Amersham International PIC, Amersham, U.K.) in the presence of TGFβ1 [2 ng/ml] at 37°C for 18 h. TGFβ is known to enhance the secretion of 66–72 kD progelatinase [Overall et al., 1989, 1991] and deposition to the matrix of an M_r 72,000 protein [Keski-Oja et al., 1986]. The media were then collected and clarified by centrifugation. The proteins were dissolved in Laemmli gel sample buffer containing 10% 2-mercaptoethanol. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to Laemmli [Laemmli, 1970], using vertical discontinuous 3.5:4.5%, 3.5:6%, or 3.5:8% sodium dodecyl sulfate-polyacrylamide gel slabs. The radiolabeled molecular weight markers were myosin (M_r 200,000), phosphorylase b (M_r 92,500), bovine serum albumin (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and lysozyme (M_r 14,300) (Amersham).

Isolation of the Pericellular Matrices

Confluent cultures of CCL-137 fibroblasts were labeled with [^{35}S]methionine in the presence of TGFβ1 [2 ng/ml] for 18–24 h. Subsequently, the cultures were extracted twice with 10 mM Tris-HCl (trishydroxymethylaminomethane) buffer, pH 8.0, containing 0.5% sodium deoxycholate and 1 mM PMSF on an ice bath [Hedman et al., 1979; Keski-Oja and Vaheri, 1982]. The substratum-bound proteins were extracted with Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography. The matrix preparations contain a reproducible number of defined polypeptides [see Keski-Oja and Vaheri, 1982; Laiho et al., 1986a].

Digestion of Fibroblast Matrix-Associated and Soluble Proteins by Tryptase and Inhibition of Tryptase Activity

For the digestion experiments, conditioned medium of human fibroblasts (CCL-137) was collected and the pericellular matrices were isolated as described above. Matrix preparations were washed twice with 100 mM Tris-HCl buffer, pH 7.5 (10 min + 1 h at 37°C), and the proteinases were applied onto them in 1 ml of 100 mM Tris-HCl buffer, pH 7.5, followed by incubation at 37°C for 20 min to 3 h as indicated. After digestion the matrices were washed with PBS (phosphate buffered saline; 2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8 mM Na_2HPO_4), the reaction was terminated with LSB (Laemmli sample buffer) [Laemmli, 1970], and the samples were analyzed in SDS-PAGE under reducing conditions. Samples of conditioned medium were digested at 37°C for 12 h and the digestion was terminated by adding Laemmli sample buffer. The samples were then analyzed by SDS-PAGE followed by autoradiography. For analysis of the gelatin binding proteins, 1 ml aliquots of [^{35}S]methionine labeled conditioned medium of CCL-137 cells were incubated with 100 μl of 50% gelatin-Sepharose for 45 min at room temperature and washed twice with 100 mM Tris-HCl buffer, pH 7.5. The gelatin-bound proteins were then treated with tryptase for 2–12 h at 37°C. The incubation was terminated by adding Laemmli gel sample buffer. The samples were then analyzed by SDS-PAGE followed by autoradiography. In all digestions with tryptase, heparin [20 μg/ml] and BSA [20 μg/ml] were added in the reaction mixture to stabilize the enzyme [Harvima et al., 1988a].

Human mast cell tryptase is inhibited by a number of different proteinase inhibitors including diisopropyl fluorophosphate, leupeptin, and benzamidine, but not by human plasma or the common trypsin inhibitors soybean trypsin inhibitor or Lima bean trypsin inhibitor [Harvima et al., 1988a]. To exclude the possibility of contaminating proteases the effects of proteinase inhibitors were analyzed on the cleavage of M_r 72,000 protein as follows: aliquots of tryptase (final concentration 15 nM) were preincubated with different proteinase inhibitors (final concentrations: aprotinin [200 IU/ml], SBTI [0.1 mg/ml], EDTA [1 mM], antipain [0.1 mg/ml], leupeptin [0.1 mg/ml], p-aminobenzamidine [10 mM], or PMSF [4 mM]) at 37°C for 12 h followed by incubation with radiolabeled conditioned medium of human lung fibroblasts followed by SDS-PAGE analysis and autofluorography.

Zymographic Assay for Gelatinolytic Activity

Analysis for gelatinolytic activity was carried out as follows: Conditioned medium from CCL-137 cells was collected and concentrated 100-fold with Micro-ProDiCon protein dialysator/concentrator (Bio-Molecular Dynamics, Beaverton, OR). Samples of the concentrate (100 μl) were treated with the indicated proteinases at 37°C for 12 h. Gelatinolytic activity was then assayed essentially as described by Hibbs et al. [1985]. Nonreduced protein samples were separated by electrophoresis using discontinuous 3.5: 4.5% polyacrylamide gel slabs containing 3 mg/ml gelatin at 4°C. After electrophoresis the gels were washed twice with 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100 (v/v), pH 7.6, for 15 min to remove SDS, followed by a brief rinsing in washing buffer without Triton X-100. The gels were then incubated at 37°C for 6 h to 4 days in 50 mM Tris-HCl buffer containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100, 0.02% NaN_3, pH 7.6. The M_r 72,000 form of gelatinase is known to be activated by SDS in the gelatinolysis gel [see Woessner, 1991]. The digestion was terminated by 10% acetic acid followed by staining with Coomassie Brilliant Blue and destaining with 10% acetic acid. Zones of enzymatic activity were seen as negatively stained bands.

Immunoblotting Analysis

For the immunoblotting analysis pericellular matrices of human fibroblasts (CCL-137) were isolated and treated with tryptase (6 nM, at 37°C for 5 h) as described above and subjected to SDS-PAGE under reducing conditions. Proteins were then transferred electrophoretically to nitrocellulose membranes for 2 h at 48 mA. Membranes were saturated with 5% milk in PBS/Triton X-100 (0.5%) and incubated with antibodies in 0.5 M Tris-HCl buffer, pH 7.4, containing 0.05 M EDTA, 1.5 M NaCl, 0.05% Tween 20. After several washes with the same buffer the bound antibodies were detected using peroxidase-conjugated anti-immunoglobulins (Dakopatts, Cøpenhagen, Denmark) and enhanced chemiluminescence (ECL) Western blotting detection system (Amersham).

RESULTS

Pericellular M_r 72,000 Protein Is a Substrate for Human Mast Cell Tryptase

To investigate the effects of tryptase on the M_r 72,000 protein, confluent cultures of fibroblasts were labeled with [^{35}S]methionine. Conditioned medium was then collected and pericellular matrices were prepared. Medium and matrix preparations were then digested with tryptase and analyzed by SDS-PAGE and autoradiography. Analysis of the autoradiograms indicated that a major effect of tryptase on the matrices was the cleavage of the M_r 72,000 protein to an M_r 62,000 form (Fig. 1A). A minor M_r 59,000 protein was frequently seen in the autoradiograms. The digestion was both time and dose dependent and was noticeable even at 0.06 nM concentration of the enzyme and within 15 min. The digestion of the M_r 72,000 protein was, however, not quantitative even at the highest tryptase concentration used and after long incubation time. This may be due to the presence of another protein of M_r 72,000 that co-migrates with the M_r 72,000 protein under reducing conditions [Overall et al., 1989]. In addition, the amount of fibronectin was reduced in the matrix in a tryptase dose-dependent manner (see below).

To analyze the effects of tryptase on the secreted radiolabeled proteins, serum-free conditioned medium of confluent fibroblast cultures labeled with [^{35}S]methionine (see above) was digested with tryptase. Autoradiograms of SDS-PAGE indicated that the M_r 72,000 protein was cleaved to an M_r 62,000 form as in the matrix (Fig. 1B). The amount of the M_r 48,000 PAI-1 in the matrix or medium preparations was not reproducibly affected by tryptase, suggesting that PAI-1 does not act as its substrate. Specifically, cleavage of PAI-1 to the M_r 4,000 lower molecular weight form, obtained by plasminogen activators, was not seen in the matrix or in the medium (data not shown).

To identify the M_r 72,000 protein and its M_r 62,000 cleavage product as gelatinase/type IV collagenase, pericellular matrices of fibroblasts were treated with tryptase and the samples were analyzed by immunoblotting with an antibody raised against a synthetic peptide corresponding to amino acid residues 472–490 in the M_r 72,000 type IV collagenase. In the control matrix the M_r 72,000 protein was the only major detectable protein, and the amount of the M_r 72,000 protein was significantly higher than that of the M_r 62,000 form. Only a faint band corresponding to M_r 62,000 form and no band corresponding to M_r 59,000 form was detected. Analysis of the tryptase treated matrix indicated cleavage of the M_r 72,000 protein to M_r 62,000 and M_r 59,000 forms (Fig. 1C).

Inhibition of Tryptase Activity by Proteinase Inhibitors and EDTA

We analyzed the effects of proteinase inhibitors and EDTA on the cleavage of radiolabeled M_r 72,000 protein (see Materials and Methods)

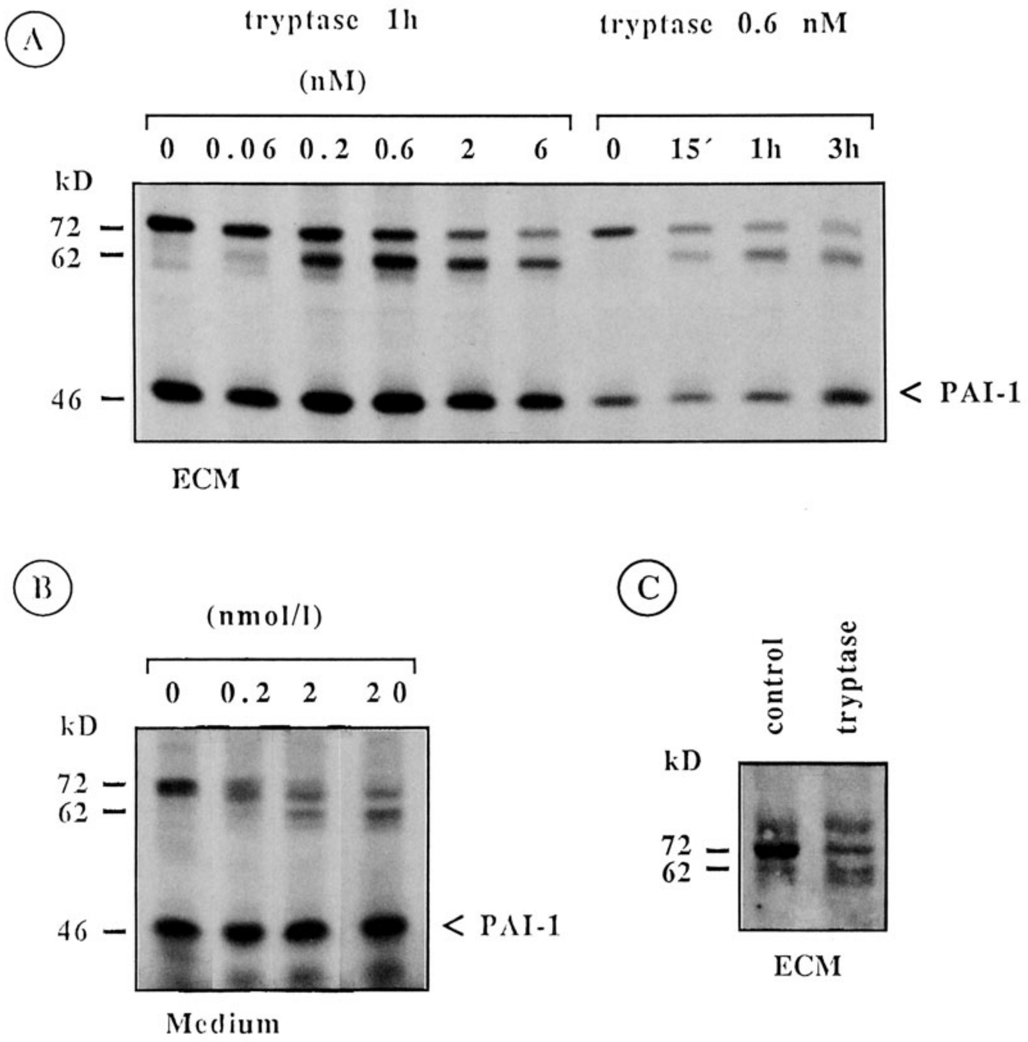

Fig. 1. Digestion of M_r 72,000 protein by human mast cell tryptase. **A:** Extracellular matrix (ECM). Confluent cultures of fibroblasts were labeled with [^{35}S]methionine in the presence of TGFβ1 (2 ng/ml) for 18 h. The pericellular matrices were then isolated and the matrices and aliquots of conditioned medium were digested with tryptase (0.06–6 nM) at 37°C for 15 min to 3 h. After the digestion the samples were dissolved in Laemmli gel sample buffer and analyzed by 4.5% SDS-PAGE under reducing conditions. Autoradiogram of [^{35}S]methionine labeled proteins is shown. The migration of PAI-1 is indicated on the right and the approximate molecular weights of the radiolabeled proteins are shown on the left. Samples for time and dose dependence of tryptase action are derived from different experiments, which explains the difference in the amounts of radioactivity. **B:** Medium. Conditioned medium from labeled cells was collected (see A) and digested with tryptase (0.2–20 nM) at 37°C for 1 h. After the digestion the samples were dissolved in Laemmli gel sample buffer and analyzed by 4.5% SDS-PAGE under reducing conditions. Autoradiogram of [^{35}S]methionine labeled proteins is shown. **C:** Immunoblotting analysis of ECM. To identify the M_r 72,000 protein and its cleavage products as gelatinase/type IV collagenase, pericellular matrices of fibroblasts were treated with tryptase (6 nM) at 37°C for 5 h and the samples were analyzed by 4.5% SDS-PAGE. The proteins were then electrophoretically transferred to nitrocellulose and immunostained with an antibody raised against a synthetic peptide corresponding to amino acid residues 472–490 in the M_r 72,000 type IV collagenase. Two cleavage products of M_r 62,000 and M_r 59,000 are seen [for molecular weights see Overall et al., 1989]. Relevant parts of the gels are shown.

by SDS-PAGE and autofluorography of the gels. It was found that the cleavage of the M_r 72,000 protein could be partially inhibited by antipain, leupeptin, p-aminobenzamidine, and PMSF but not by aprotinin, SBTI, or EDTA (Fig. 2), which is in accordance with the reports of inhibition of tryptase activity [Harvima et al., 1988a]. Even a higher concentration of EDTA (30 mM) could not inhibit the cleavage, indicating that no metalloproteinases are involved as intermediate activators (data not shown). The mechanism of the cleavage of the M_r 72,000 protein thus appears to differ from the activation of interstitial collagenase by tryptase, which seems to be depen-

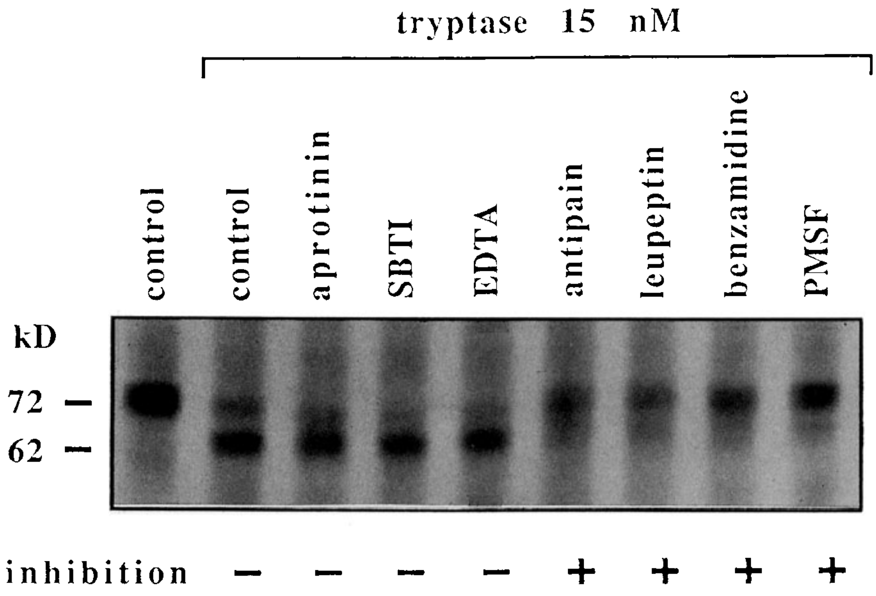

Fig. 2. Inhibition of tryptase effect on M_r 72,000 protein by specific inhibitors of tryptase. Confluent cultures of fibroblasts were labeled with [^{35}S]methionine in the presence of TGFβ1 [2 ng/ml]. Conditioned medium was collected after 20 h incubation. Aliquots of human mast cell tryptase (final concentration 15 nM) were preincubated with different proteinase inhibitors (final concentrations: aprotinin [200 IU/ml], SBTI [0.1 mg/ml], EDTA [1 mM], antipain [0.1 mg/ml], leupeptin [0.1 mg/ml], p-aminobenzamidine [10 mM], or PMSF [4 mM]) at 37°C for 12 h, followed by incubation with 200 μl aliquots of CCL-137 conditioned medium at 37°C for 3 h. Untreated conditioned medium and tryptase digested conditioned medium without inhibitors were used as controls. After digestion the samples were dissolved in Laemmli sample buffer and analyzed in 4.5% SDS-PAGE under reducing conditions. Inhibition of the cleavage of the M_r 72,000 protein is indicated at the bottom of the figure. The approximate molecular weights of the radiolabeled proteins are shown on the left. Relevant part of the gel is shown.

dent on stromelysin (MMP-3) activation [Gruber et al., 1989].

Native and Tryptase Treated Forms of the M_r 72,000 Protein Have Gelatinolytic Activity in Gelatin Zymography Assays

To examine the possible gelatinolytic activity of the M_r 72,000 and M_r 62,000 proteins, samples of fibroblast conditioned medium were treated with tryptase and other proteinases followed by analysis for gelatinolytic activity (see Materials and Methods). Inspection of the gelatinolysis areas showed only a single band of M_r 72,000 of enzymatic activity prior to treatment with tryptase. Digestion with tryptase, thrombin, and chymase produced another enzymatically active band of M_r 62,000 (Fig. 3A: results of chymase digestion are not shown). The appearance of the lower molecular weight form was dependent both on time (gel not shown) and the dose of tryptase. In accordance with our results of radiolabeled proteins even long incubation with high concentrations of tryptase did not lead to quantitative digestion of the higher molecular weight form under these assay conditions. This is in accordance with Lefebvre et al. [1991] who found that digestion of chondrocyte conditioned medium with trypsin caused only partial conversion of M_r 72,000 gelatinolytic activity to an M_r 62,000 form.

To further assess the question whether the gelatinolytic protein found in gelatin zymography was gelatinase/type IV collagenase, samples of recombinant M_r 72,000 type IV collagenase were treated with or without tryptase. Analysis of the gelatin zymogram showed partial cleavage of the recombinant protein to the lower molecular weight form (Fig. 3B).

Digestion of the Pericellular Proteins by Chymase: Effects on the 72,000 Dalton Protein and Fibronectin

To analyze the effects of the other major mast cell derived enzyme, chymase (that is recognized as an activator of latent collagenase), on the pericellular matrix-associated proteins of human fibroblasts, especially on the M_r 72,000 protein and fibronectin [Vartio et al., 1981], and to compare its effects to those of tryptase, radiolabeled isolated pericellular matrices were

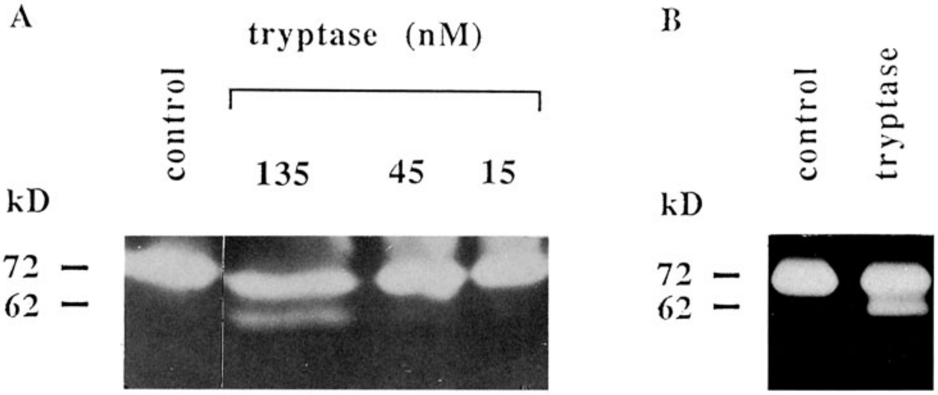

Fig. 3. Gelatin zymograms of concentrated fibroblast conditioned medium and recombinant M_r 72,000 gelatinase/type IV collagenase. **A:** Confluent cultures of fibroblasts were cultivated in the presence of TGFβ1 (2 ng/ml). Conditioned medium was then collected and concentrated 100-fold as described (see Materials and Methods). Samples of the concentrated conditioned medium were then treated with tryptase (concentrations as indicated) at 37°C for 12 h. The reaction was terminated by adding nonreducing Laemmli gel sample buffer. The proteins were then separated by 4.5% SDS-PAGE containing 3 mg/ml gelatin. After electrophoresis SDS was washed away and the gel was incubated at 37°C for 12 h. Areas of gelatinolytic activity were visualized by staining the gel with Coomassie Brilliant Blue stain. Relevant part of the gel is shown. **B:** A sample of recombinant M_r 72,000 type IV collagenase was treated with tryptase (30 nM) and incubated with untreated control at 37°C for 4 h. The reaction was terminated by adding nonreducing Laemmli gel sample buffer. The gelatinolytic activity was then analyzed as described above. Relevant part of the gel is shown.

treated with chymase and analyzed by SDS-PAGE under reducing conditions followed by autofluorography. It was found that a similar cleavage of the M_r 72,000 protein by chymase occurred both in a time- and dose-dependent manner (Fig. 4). Degradation of fibronectin was also noticed in the autoradiograms [data not shown; see Vartio et al., 1981]. In addition, it was found that the amount of matrix-bound PAI-1 was decreased in the matrix by chymase in a time- and dose-dependent manner, possibly because of its release from the matrix (Fig. 4, highest concentrations). Comparison of the autoradiograms indicated that the cleavage products of M_r 72,000 protein of tryptase and chymase were of approximately similar size (Figs. 1, 4). The M_r 62,000 chymase cleavage product was also capable of degrading gelatin in the gelatin zymography assay (data not shown).

Fibronectin Is a Substrate for Human Mast Cell Tryptase

To analyze the effect of tryptase on the major proteinase sensitive extracellular matrix protein fibronectin, isolated fibroblast matrices were treated with tryptase and alterations of pericellular fibronectin were observed in autofluorograms of SDS-PAGE. It was found that the amount of fibronectin in the matrix was decreased by tryptase in a dose-dependent manner (Fig. 5A). Analysis of the radiolabeled trichloroacetic acid (10% final concentration) precipitates of matrix digestion supernatants revealed dose-dependent release from the extracellular matrix of fibronectin and fibronectin degradation products (gel not shown).

To analyze the effects of tryptase on soluble fibronectin, the radiolabeled gelatin binding proteins of fibroblast medium were bound to gelatin-Sepharose particles and treated with tryptase [6–60 nM] for 2–12 h. Analysis by 8% SDS-PAGE under reducing conditions indicated that the amount of M_r 220,000 fibronectin was decreased during tryptase digestion (Fig. 5B). Cleavage of gelatin-bound M_r 72,000 protein to M_r 62,000 form was inefficient under these conditions. The probable reason for this is that gelatin interferes with the interaction of tryptase with the M_r 72,000 protein and is thus anticatalytic. Analysis of the degradation of dimeric fibronectin under nonreducing conditions in a 4.5% SDS-PAGE showed that both the dimeric M_r 440,000 and monomeric 220,000 forms of fibronectin were degraded resulting in the appearance of lower molecular weight fragments (Fig. 6). After the digestion, fragments of M_r 180,000, 160,000, and 27,000 were observed in the autoradiograms. The appearance of the fragments correlated with the disappearance of fibronectin, and their sizes were similar to the

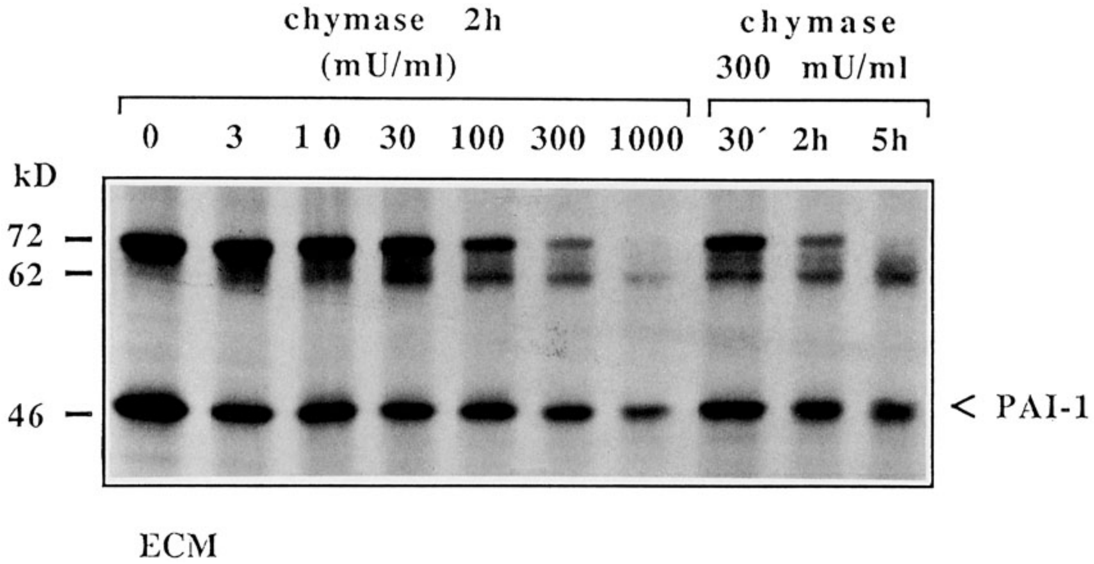

Fig. 4. Digestion of the M_r 72,000 protein by rat chymase. Confluent cultures of fibroblasts were labeled with [^{35}S]methionine in the presence of TGFβ1 as described in Materials and Methods. Conditioned medium was collected after 20 h incubation. Aliquots of conditioned medium were treated with chymase [3–1,000 mU/ml] at 37°C for 30 min to 5 h. After digestion the samples were dissolved in Laemmli sample buffer and analyzed in 4.5% SDS-PAGE under reducing conditions. Autoradiogram of [^{35}S]methionine labeled proteins is shown. Migration of PAI-1 is indicated on the right: the approximate molecular weights of the radiolabeled proteins are shown on the left. Note that the amount of PAI-1 in the matrix is decreased. Relevant part of the gel is shown.

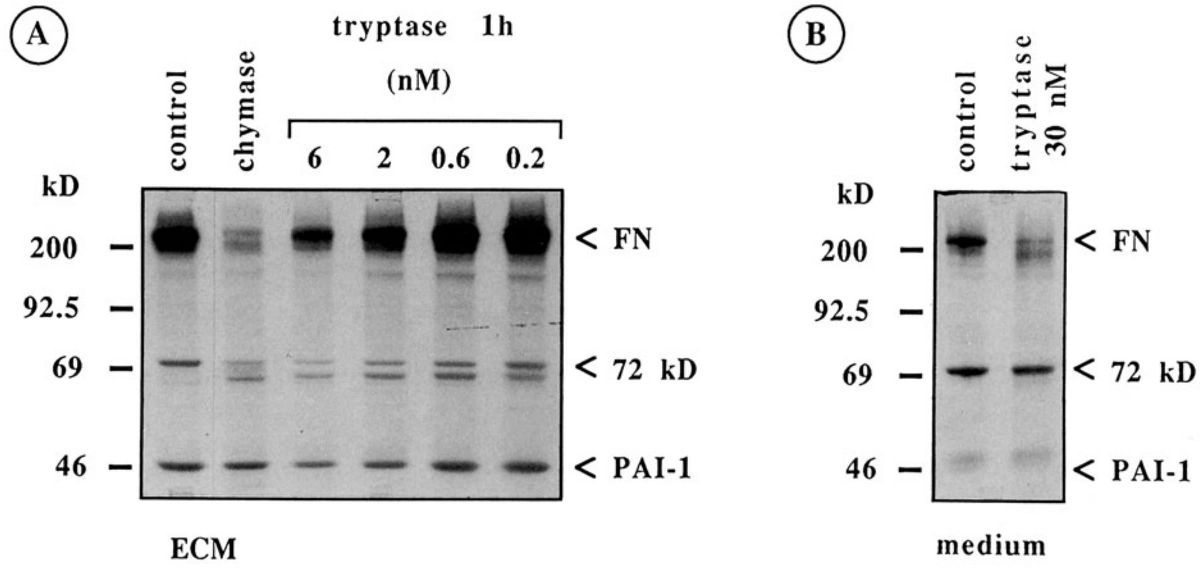

Fig. 5. Digestion of pericellular matrix and gelatin binding proteins of conditioned medium of fibroblasts by tryptase. **A:** Extracellular matrix (ECM). Confluent cultures of fibroblasts were labeled with [^{35}S]methionine in the presence of TGFβ1 [2 ng/ml]. The pericellular matrices were isolated and matrices were digested with tryptase [0.2–6 nM] or chymase [0.2 U/ml] at 37°C for 1 h. The digestion was terminated by Laemmli sample buffer and the samples were analyzed by 8% SDS-PAGE under reducing conditions followed by autoradiography. **B:** Medium. Conditioned medium from labeled cells was collected (see A). Then 1 ml aliquots of the conditioned medium were incubated with 100 μl of 50% gelatin-Sepharose for 45 min, washed twice with 100 mM Tris-HCl buffer, pH 7.6, and treated with tryptase (30 nM) at 37°C for 2 h. After digestion samples were dissolved in Laemmli sample buffer and analyzed by 8% SDS-PAGE under reducing conditions followed by autoradiography. The migration of fibronectin, the M_r 72,000 protein, and PAI-1 are shown on the right, and the sizes of molecular weight markers are indicated on the left. Note the decrease of the amount of matrix associated fibronectin (A) and the degradation of gelatin bound fibronectin (B) after tryptase treatment. ECM, isolated extracellular matrices; FN, fibronectin.

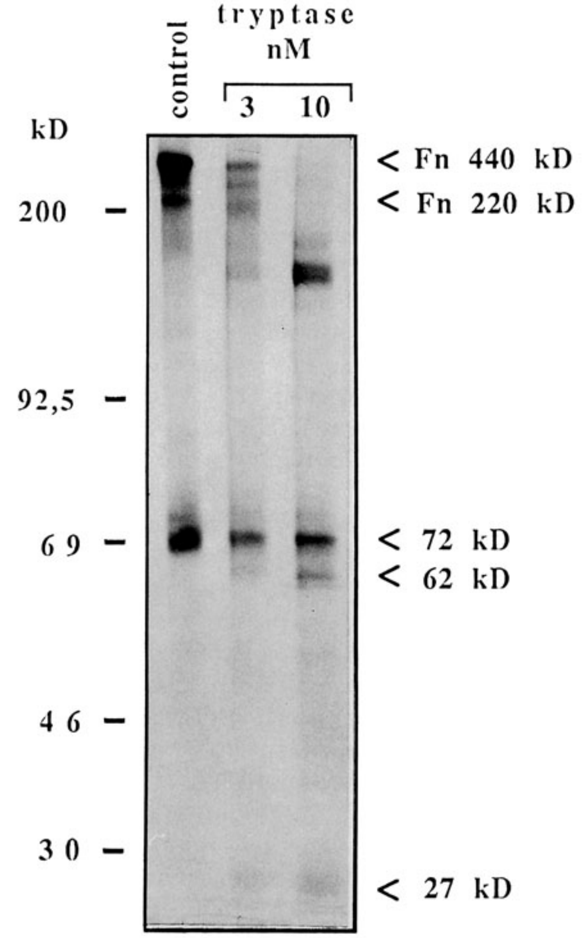

Fig. 6. Analysis of the tryptase digestion products of the gelatin binding proteins of human fibroblast medium under nonreducing conditions. Confluent cultures of fibroblasts were labeled with [^{35}S]methionine in the presence of TGFβ1 (2 ng/ml) and the conditioned medium was collected as in Fig. 5B. Then 1 ml aliquots of the conditioned medium were incubated with 100 μl 50% gelatin-Sepharose for 45 min, washed twice with 100 mM Tris-HCl buffer, pH 7.5, and treated with tryptase (3–10 nM) at 37°C for 12 h. After digestion the samples were dissolved in Laemmli sample buffer and analyzed in 4.5% SDS-PAGE under nonreducing conditions followed by autoradiography. The migration of the M_r 440,000 dimeric and 220,000 monomeric forms of fibronectin, the M_r 72,000 and 62,000 proteins, and a M_r 27,000 fibronectin fragment are shown on the right, and the mobility of the molecular weight markers is indicated on the left. Note the disappearance of fibronectin and the appearance of M_r 180,000, 160,000, and 27,000 fragments of fibronectin after tryptase treatment. FN, fibronectin.

sizes of fibronectin fragments generated by certain other proteinases [Vartio et al., 1981; Quigley et al., 1987; Gold et al., 1989; see Yamada, 1989]. After prolonged digestion (Fig. 6: 12 h vs. 2 h in Fig. 5B) partial cleavage of gelatin-bound M_r 72,000 protein to an M_r 62,000 form was observed. Under nonreducing conditions type IV collagenase/gelatinase migrates as an M_r 66,000 protein [Vartio and Vaheri, 1981] separately from an unrelated M_r 72,000 protein described by Overall et al. [1989].

To exclude the possibility that the M_r 62,000 digestion product would be a fragment of fibronectin, both fibroblast conditioned medium and purified human plasma fibronectin were treated with tryptase (12 nM) at 37°C for 5 h and analyzed by immunoblotting using polyclonal anti-fibronectin antibodies (see Materials and Methods). The analysis revealed immunoreactive fragments of M_r 180,000 and 160,000 while no immunoreactivity at the M_r 62,000 region was seen (data not shown). In addition, analysis of purified plasma fibronectin and its digestion products by the employed gelatinolysis assay did not reveal any lysis zones further indicating that the M_r 72,000 and M_r 62,000 proteins are distinct from fibronectin [see also Vartio and Vaheri, 1981].

DISCUSSION

Most cells have an ability to pass through extracellular matrices and basement membranes. In this process the cells need to degrade the network of type IV collagen, the major component of basement membranes. Specific basement membrane collagen degrading enzymes, type IV collagenases are metalloproteinases that are produced by a variety of normal and malignant cells [Liotta et al., 1980; Collier et al., 1988]. Type IV collagenase is identical to human fibroblast gelatinase, and it is also referred to as matrix metalloendoproteinase-2 (MMP-2) [Collier et al., 1988; for nomenclature see also Woessner 1991].[2] The M_r 72,000 type IV collagenase/gelatinase is also a regular component of human plasma [Vartio and Baumann, 1989]. It is homologous to some other metalloproteinases, collagenase (MMP-1) and stromelysin (MMP-3) [Okada et al., 1986; Höyhtyä et al., 1988; Collier et al., 1988], but the substrate specificities of the three enzymes are somewhat different. Type IV collagenase/gelatinase produced by fibroblasts is immunologically and structurally distinct from that secreted by macrophages and polymorphonuclear leukocytes, the molecular weights being M_r 66,000–72,000 and 94,000–97,000, respectively [Murphy et al., 1989; Huhtala et al., 1991].

[2]The International Union of Biochemistry has recently recommended the name gelatinase A for the M_r 72,000 gelatinase, and gelatinase B for the M_r 92,000 gelatinase.

Their substrate specificities are, however, identical. Related gelatinase activities of M_r 130,000 and 225,000 have also been found [Hibbs et al., 1985; Gold et al., 1989].

Secretion of M_r 72,000 type IV collagenase is characteristic of malignant cells [Liotta et al., 1981], and its expression and activity correlates to the invasive and metastatic properties of tumor cells [Liotta et al., 1980; Ura et al., 1989]. This enzyme, however, is secreted as an inactive proenzyme, and mechanisms of its activation in vivo are poorly known. Previously it has been found that the activation of the proenzyme forms of metalloendoproteinases can be accomplished in vitro by treatment with organomercurial compounds [Cawston et al., 1981; Stetler-Stevenson et al., 1989], sulphydryl reagents [Werb and Reynolds, 1974] and disulfides [Tschesche and McCartney, 1981], or with some proteinases such as trypsin, chymotrypsin, plasmin, kallikrein, cathepsin B, and some endogenous neutral and serine proteinases [Stricklin et al., 1983; Vaes, 1972; Werb et al., 1977; Eechout and Vaes, 1977; Salo et al., 1982]. It has been reported that type IV collagenase can also be activated by trypsin can also be activated by trypsin and organomercurial compounds [Liotta et al., 1981; Collier et al., 1988]. Organomercurial treatment leads to the autocatalytic cleavage and activation of the proenzyme to a smaller form, which could correspond to the cleavage of M_r 72,000 protein to an M_r 62,000 form observed in the present work [Stetler-Stevenson et al., 1989]. In addition, a minor degradation product of M_r 59,000 has been detected. This is presumably an autodigestion product of type IV collagenase. On the other hand, it has been reported that proteolytic degradation of latent type IV collagenase by trypsin and plasmin do not activate the latent basement membrane collagenase activity [Okada et al., 1990]. While the reaction conditions vary, it is evident that all proteases cannot directly activate the 72 kDa type IV collagenase. However, processing by different proteases is likely to alter the protein to a form that can be more readily activated by other mechanisms unknown so far.

Mast cells are present in large numbers in dermal skin, in the mucosa and submucosa of the gastrointestinal and respiratory tracts, and in bronchial lumen as free cells [Patterson et al., 1977; Soter et al., 1978; Befus et al., 1985; Shanahan et al., 1987]. In their cytoplasmic granules mast cells have various types of bioactive substances, including biogenic amines, neutral proteases, acid hydrolases, oxidative enzymes, chemotactic factors, and proteoglycans. Major neutral proteases present in mast cell granules are tryptase and chymase [Schwartz et al., 1987]. Interaction of mast cells with multivalent antigens for the membrane bound IgE antibodies or various nonspecific stimuli may lead to the activation of the mast cell. Tryptase and chymase may play a crucial role in the process of degranulation of mast cells, as their inhibitors also inhibit histamine release induced by anti-IgE challenge [Kido et al., 1985]. Tryptase has been found to activate latent interstitial collagenase from synovial fluid and cultured synoviocyte conditioned medium [Gruber et al., 1988]. This activation, however, seems to depend on the activation of prostromelysin, which then activates the latent interstitial collagenase [Gruber et al., 1989]. In the present work we found that tryptase could use as its substrate the M_r 72,000 gelatinolytic protein probably identical to M_r 72,000 type IV collagenase/gelatinase. This cleavage was not dependent on stromelysin as it could not be inhibited by EDTA. Processing by tryptase to the M_r 62,000 form that can be activated in the indicator gel system by SDS into a proteolytically active gelatinase indicates that the cleavage does not inactivate the enzyme. However, the current experimentation does not provide evidence for the activation of the M_r 72,000 gelatinase/type IV collagenase into the type IV collagen degrading form. This type of proteolytic processing may present an important physiological event in vivo that could occur in mast cell mediated events like acute and chronic inflammation, allergic reactions, and their consequences.

The C-terminus of the 72 kDa gelatinase can also be removed by proteases, after which the enzyme retains its gelatinolytic activity. The affinity of the major gelatinase inhibitor TIMP-2 to the truncated gelatinase is considerably decreased [Howard et al., 1991, Fridman et al., 1992]. Thus also C-terminal cleavage may contribute to enhanced enzymatic activity. Our data does not allow speculation of the cleavage site.

Adherent cells secrete a number of glycoproteins and proteoglycans that are constituents of the extracellular matrix. For example, fibroblastic cells produce distinct gelatin binding proteins of M_r 440,000 (fibronectin) [see Vartio, 1982], M_r 66,000–72,000 (gelatinase/type IV procollagenase) [Vartio and Vaheri, 1981] and M_r

21,000 [Keski-Oja et al., 1986]. Of these fibronectin and gelatinase have been identified as constituents of the pericellular matrix [Hedman et al., 1979; Keski-Oja and Todaro, 1980]. The turnover of the extracellular matrix is regulated by the production and pericellular deposition of extracellular matrix components and different proteases that either degrade the ECM themselves or activate other proteinases.

In the current work we have found that the human mast cell derived enzyme tryptase has two major substrates in the extracellular matrix of human fibroblasts, fibronectin and a gelatinase. Fibronectin is susceptible to the action of a number of proteinases, and as shown earlier for chymase [Vartio et al., 1981] also tryptase was able to digest cellular fibronectin. Digestion with tryptase of fibroblast extracellular matrix proteins or secreted gelatin binding proteins resulted in the generation of M_r 180,000, 160,000, and 27,000 fragments that resemble those obtained with various proteinases [see Yamada, 1989]. The release of fibronectin from the matrix is frequently associated with the cleavage of the M_r 72,000 (66,000) protein. However, these events appear to be independent of each other [Keski-Oja and Vaheri, 1982]. As a difference between the action of these proteinases it was found that the amount of matrix-bound PAI-1 was decreased by chymase but not by tryptase. An M_r 72,000 protein, evidently related to gelatinase/type IV procollagenase, was proteolytically processed by tryptase to an M_r 62,000 form. The identity of these proteins was confirmed by immunoblotting with an antipeptide antibody against type IV collagenase. Recombinant M_r 72,000 type IV collagenase was also cleaved by tryptase to a comparable M_r 62,000 fragment. Our results suggest that like in the case of chymase the major functions of human mast cell tryptase are probably associated with cell invasion and degradation of pericellular structures.

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