Tumor Promoter-stimulated M, 92,000 Gelatinase Secreted by Normal and Malignant Human Cells: Isolation and Characterization of the Enzyme from HT1080 Tumor Cells

Ute M. Moll, Gary L. Youngleib, Karen B. Rosinski, and James P. Quigley

ABSTRACT

A M, 92,000 metalloprotease, originally observed in neutrophils, has been found to be secreted by various normal and malignant cells of fibroblastic, hematopoietic, and epithelial origin. The responsiveness of the various cell types to the tumor promoter phorbol ester (phorbol myristate acetate) to secrete this enzyme and a corresponding M, 72,000 gelatinase has been determined using gelatin zymograms. The latent zymogen form of the M, 92,000 enzyme has been purified from phorbol myristate acetate-stimulated HT1080 human fibrosarcoma cells using sequential gelatin-Sepharose affinity chromatography and gel filtration. Selective elution from gelatin-Sepharose allows for autoactivation of the proenzyme to active M, 83,000 and M, 75,000 species. At physiological pH, the enzyme rapidly degrades gelatin into small fragments and slowly cleaves native type V collagen at an apparent single site. Native type IV collagen is degraded to a much lesser extent. The NH2-terminal amino acid sequence of the M, 92,000 proenzyme has been determined and is distinct from the M, 72,000 gelatinase/type IV collagenase which is constitutively produced by fibroblasts. The M, 92,000 enzyme is also immunologically distinct from the M, 72,000 enzyme but immunologically cross-reactive with the neutrophil, high molecular weight gelatinase. The M, 92,000 enzyme constitutes a distinct member of the matrix metalloprotease family. Its substrate specificity implies a broad physiological role, acting on basement membrane type V collagen as well as on denatured (gelatinized) collagens and thus may be involved in the invasive and migratory phenotype of human cells.

INTRODUCTION

The invasive and metastatic ability of neoplastic cells depends on the catalytic degradation of extracellular matrix and basement membrane. This process is mediated by secreted proteolytic enzymes, of which metalloproteases, serine proteases, and cathepsins appear to be the most important (1). The final catalytic effectors of extracellular proteolysis seem to be enzymes from the metalloprotease family. Members of this family include interstitial (types I–III) collagenase (2, 3), a M, 72,000 gelatinase (4, 5) which degrades type IV and V collagen, a M, 92,000 gelatinase originally described as a neutrophil gelatinase (6) which also degrades native type V collagen, stromelysin (7, 8), which degrades proteoglycans, and PUMP-1, a newly detected member (9). Metalloproteases are capable of degrading most fibrillar and amorphous matrix proteins. In normal morphogenesis and neoplastic invasion, metalloproteases are regulated at multiple levels. Their synthesis can be regulated by interleukin 1 (10) and transforming growth factor β (11, 12). Furthermore, interstitial collagenase and stromelysin can be induced by phorbol esters. This inducibility is conferred by a phorbol ester-responsive element in the promoter regions of these genes (13). Metalloproteases are secreted as latent zymogens and their activation has been shown to occur via complex proteolytic cascade systems involving urokinase-type plasminogen activator (14–16), other metalloproteases (17, 18), and autoactivation (19). In many cell systems, the active enzymes are controlled by cosecreted TIMPs (20–24).

A strong correlation between the production of basement membrane-degrading metalloproteases and metastasis has been shown for various transformed cell lines (25–28). For example, transfection of c-Ha-ras into fibroblasts induces these cells to secrete high levels of type IV collagenase and concomitantly become spontaneously metastatic in nude mice. Cotransfection with the suppressing Ad2-E1a gene results in tumorigenic but nonmetastatic cells which have lost their ability to produce type IV collagenase (27). In many of these in vitro and in vivo studies, total gelatinase or type IV collagenase activity was measured and it was assumed that the previously described M, 72,000 gelatinase/type IV collagenase was the enzyme responsible for the observed cleavage of type IV collagen. However, in a recent preliminary study, a novel, metastasis-related M, 92,000 metalloprotease was reported (29). The M, 92,000 activity, as measured by gelatin zymograms, was markedly increased in highly metastatic, murine carcinoma sublines when compared to poorly metastatic sublines (29). We have observed that the human fibrosarcoma cell line HT1080 produces large amounts of M, 92,000 enzyme, whereas normal human fibroblasts are reported to secrete little or no enzyme (5, 29). Both cell types, however, produce comparable amounts of the M, 72,000 enzyme. We undertook this study to purify and characterize the M, 92,000 metalloprotease from HT1080 cells. After the work was completed, a parallel investigation of a M, 92,000 enzyme in SV40-transformed human lung fibroblasts was reported (30).

MATERIALS AND METHODS

Reagents. Gelatin-Sepharose and Superose 12 resin were purchased from Pharmacia Fine Chemicals. APMA, 3-(cyclohexylamino)-1-propanesulfonic acid, Me2SO, gelatin, BSA, and the inhibitors EDTA, 1,10-phenanthroline, PMSF, and dithiothreitol were purchased from Sigma. Rat tail type I collagen was obtained from Dr. J-M. Chen, Thomas Jefferson University, Philadelphia, PA; 3H-rat tail type I collagen (0.98 mCi/mg) was purchased from Du Pont-New England Nuclear; murine type IV collagen (Engelbreth-Holm-Swarm) was purchased from Collaborative Research, Inc., and Bethesda Research Lab.
oratories; bovine bone type V collagen was a gift from Dr. C. Niyibizi, University of Washington, Seattle, WA. Human fibronectin was purchased from Collaborative Research Inc. Murine laminin (Engelbreth-Holm-Swarm) was a gift from Dr. H. Kleinman, NIH, Bethesda, MD. Sheep anti- pi-g neutrophil gelatinase IgG and sheep anti-human TIMP IgG were gifts from Dr. G. Murphy, Strangeways Laboratories, Cambridge, United Kingdom (31, 32).

Cell Culture. The human fibrosarcoma cell line HT1080 was cultured in MEM containing 7% FBS. Human foreskin fibroblasts and human synovial fibroblasts were grown in 7% FBS supplemented Eagle’s MEM. Freshly harvested human neutrophils were maintained in serum free MEM containing PMA (100 ng/ml) for 2 h. A low and a high metastatic variant of the human squamous cell carcinoma line HEP-2 (33, 34) were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS. The human monocytic leukemia strain U-937 and the human promyelocytic leukemia strain HL60 (gifts from Dr. Howard Fleit, SUNY at Stony Brook) were cultured in RPMI 1640 containing 7% FBS. For screening experiments, all cell lines except the human neutrophils were grown in serum-free medium with and without PMA (100 ng/ml) and the conditioned medium was harvested after 24 h from near confluent cultures. Aliquots were standardized for equivalent cell numbers and assayed on zymograms. For the purification of M, 92,000 enzyme from conditioned medium, HT1080 cells were grown to confluence, washed 3 times in phosphate buffered saline and changed to serum free MEM containing PMA (100 ng/ml). Three to four 24-h harvests were collected before the cells were split again.

Enzyme Purification. The M, 92,000 metalloprotease was purified using a 3-step protocol. Serum-free conditioned medium was precipitated overnight with (NH₄)₂SO₄ to 70% saturation, pH 7.0, at 4°C. The precipitate was centrifuged for 60 min at 10,000 rpm, redissolved in 1/10 the volume of the conditioned medium and dialyzed in Brij buffer (0.5 M NaCl-5 mM CaCl₂-50 mM Tris-0.05% NaN₃, pH 7.6). The material was applied to a gelatin-Sepharose column (0.95 x 17 cm) which had been equilibrated in Brij buffer as described previously (6). After the column was washed in the same buffer, gelatin-binding proteins were eluted with 7.5% Me₂SO buffer (1 M NaCl-50 mM Tris-5 mM CaCl₂-7.5% Me₂SO-0.05% NaN₃, pH 7.6), dialyzed against Brij buffer, and pooled. The preparation was applied onto a new gelatin-Sepharose column (0.95 x 17 cm) and stepwise eluted with Me₂SO buffers (1 M NaCl-50 mM Tris-5 mM CaCl₂-0.05% Brij 35-30% Me₂SO, pH 7.6) containing 2, 4, and 7.5% Me₂SO respectively. After dialysis against Brij buffer, the eluted fractions were analyzed on silver-stained polyacrylamide gels (5-15% and 7.5%) (35) and gelatin substrate gels. The 2% Me₂SO eluted fractions, which were enriched in the M, 92,000 enzyme, were pooled, concentrated with polyethylene glycol, dialyzed against Hepes buffer (10 mM Hepes-1 M NaCl-5 mM CaCl₂-0.05% Brij-0.02% NaN₃, pH 7.6), and applied to a 0.95-x 25-cm gel filtration column (Superose 12 HR10/30) which had been equilibrated with the same Hepes buffer. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min. Apparent molecular weights were estimated using known molecular weight standards. Protein concentrations were measured as absorbance at 280 nm, by microplate Bradford assay (Bio-Rad Laboratories, Richmond, CA), and estimated from silver-stained bands after SDS-PAGE using bovine serum albumin standard curves.

Western Immunoblotting. Aliquots of the 7.5% Me₂SO elute from the first gelatin-Sepharose column, the 2% Me₂SO elute from the second gelatin-Sepharose column, and a FPLC preparation were run on a 7.5% SDS-PAGE, electroblotted, blocked with 5% nonfat milk/ PBS for 45 min, and incubated overnight at room temperature with sheep anti-pi-g neutrophil gelatinase IgG (35 mg/ml) diluted 1:300 (31) and sheep anti-human TIMP IgG (55 mg/ml) diluted 1:1000 (32). Alkaline phosphatase conjugated anti- sheep IgG (1:1000, Sigma) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) were used to develop the blots. A mouse antiserum, raised against a partially purified M, 92,000 preparation, was blotted against the 7.5% Me₂SO gelatin-Sepharose elute at a dilution of 1:500 and developed with horseradish peroxidase conjugated anti-mouse IgG (1:500; Kirkegard and Perry Laboratory) and chloronaphthol/H₂O₂.

Gelatinase Assays. Gelatinolytic activity of partially purified and purified M, 92,000 enzyme was assayed by using heat denatured (20 min at 60°C) 3H-labeled type I collagen (7000 cpm/assay) as previously described (26). Aliquots (10 µl) of purified enzyme (40 ng) were preactivated before substrate was added with 2.5 mM APMA (dissolved in Me₂SO) for 2 h at 37°C. For inhibition studies, 5 mM EDTA, 5 mM 1,10-phenanthroline, 2 mM PMSF, and 1 mM dithiothreitol were added along with the substrate.

Amino Acid Sequence Analysis. Pooled 2% Me₂SO eluates from the second gelatin-Sepharose column containing 10 and 20 µg of M, 92,000 proenzyme were run on SDS-PAGE gradient gels (5-15%) and electroblotted onto PVDF membranes (Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer free of methanol (36). The membranes were stained with Coomassie Brilliant Blue. The unactivated M, 92,000 band and the M, 29,000 band were carefully cut out and the NH₂-termini of both proteins were sequenced in an Applied Biosystem 470 gas phase sequencer. Four separate sequence determinations were carried out.

Gelatin Zymography. SDS-PAGE gels were prepared according to the method of Laemmli (37) and included copolymerized gelatin at a final concentration of 0.19 mg/ml. Enzyme-containing samples were dissolved in SDS sample buffer in the absence of reducing agents and boiled and were electrophoresed on 7.5% or 5-15% gradient gels (37). After electrophoresis, the gels were washed 2 x 30 min in 2.5% Tiron X-100 to remove SDS and incubated overnight at 37°C in 40 mM Tris-200 mM NaCl-10 mM CaCl₂, pH 7.5, and then stained in Coomassie Brilliant Blue (0.1%). Clear zones of gelatin lysis against a blue background stain indicated the presence of enzyme. For the enzyme activation studies, 2-µl samples of purified enzyme in 40 mM Tris-200 mM NaCl-10 mM CaCl₂, pH 7.5, buffer were preincubated at 37°C in 2 mM APMA for 0-6 h. In addition, some samples received 2 mM PMSF or 5 mM EDTA.

Substrate Specificity. Assays with type I and V gelatin (prepared from type I and V collagens by heating to 60°C for 20 min) as substrates (12 µg/assay) were performed at 23°C and 37°C using 16 ng of purified enzyme for up to 6 h in 50 µl reaction mixture (50 mM Tris-5 mM CaCl₂-1 mM ZnCl₂-1% Triton X-100-1 mM APMA, pH 7.6). Assays with native type IV and V collagen (15 µg/assay) were performed at 32°C using 320 ng of purified APMA activated enzyme for up to 48 h in 140 µl of reaction mixture (containing APMA). Fibronectin and laminin (20 µg/assay) were incubated with 140 ng of purified enzyme at 37°C for up to 24 h in 50 µl reaction mixture.

In a second set of experiments, pooled eluates from the first gelatin-Sepharose column were electrophoresed on 6% SDS-PAGE. After the SDS was exchanged with 2.5% Triton X-100 (3 x 30 min), the M, 92,000 band and control bands from irrelevant areas of the gel were cut out under the guidance of Coomassie stained indicator lanes containing the same material (6). Type V collagen (50 µg/assay), type IV collagen (15 µg/assay), and native type I collagen (30 µg/assay) were incubated with enzyme-containing gel slices (cut into 1-mm cubes) containing approximately 2 µg of enzyme for 48 h at 32°C in 150 µl of reaction mixture (50 mM Tris-5 mM CaCl₂-1 mM ZnCl₂-1% Triton X-100-1 mM APMA, pH 7.6). All incubations were terminated by the addition of 5 mM EDTA, the gel cubes were removed, the samples were reduced and boiled for 2 min, subjected to SDS-PAGE (7.5 and 5-15%), and stained with Coomassie Blue to detect collagen degradation products.

RESULTS

Various human cell types secrete the M, 92,000 metalloprotease, the presence of which in conditioned media was readily detected by its gelatinolytic activity in zymograms (Fig. 1). Aliquots of 24-h-conditioned media harvested from near confluent cell cultures were standardized for cell numbers and tested. The fibrosarcoma cell line HT1080 constitutively secretes the M, 92,000 enzyme and the M, 72,000 gelatinase/type IV collagenase (4, 5) in comparable amounts (Fig. 1, Lane 1).
PMA stimulation of HT1080 cultures induces the M, 92,000 enzyme by at least 2-fold but not the M, 72,000 gelatinase (Fig. 1, Lane 2). The secretion of higher molecular weight gelatinases are also induced by PMA. Among normal fibroblast strains, synovial fibroblasts secrete more M, 92,000 enzyme than human foreskin fibroblasts, but in both strains the M, 92,000 enzyme is strongly induced with phorbol ester (Fig. 1, Lanes 3–6). Both strains of normal fibroblasts secrete the M, 72,000 gelatinase approximately equal to that of HT1080 cells and like the HT1080 cells, the M, 72,000 enzyme is not significantly induced by PMA. In the squamous cell carcinoma line HEp-3, the low metastatic (Fig. 1, Lanes 7 and 8) and the high metastatic (Fig. 1, Lanes 9 and 10) variants show little difference in their M, 72,000 gelatinase production. However, the M, 92,000 enzyme is produced by the high metastatic variant, while the low metastatic strain fails to produce any significant amounts. Among the hematopoietic cell lines, the monocytic leukemic strain U937 secretes minimal amounts of M, 72,000 gelatinase and no M, 92,000 enzyme (Fig. 1, Lane 11). Upon PMA induction, both enzymes are secreted at low levels (Fig. 1, Lane 12). The promyelocytic leukemic strain HL60 produces no M, 72,000 and minimal amounts of M, 92,000 enzyme (Fig. 1, Lane 13). However, PMA causes a strong induction of M, 92,000 gelatinase, while the M, 72,000 activity becomes just detectable (Fig. 1, Lane 14). PMA stimulated human neutrophils secrete a M, 92,000 gelatinase as well as higher molecular weight enzyme complexes (of M, 130,000 and M, 220,000) (Fig. 1, lane 15) as previously shown (6). It should be noted that the adherent cell lines were near confluency, whereas the hematopoietic cell lines grew in suspension which makes these two groups not directly comparable.

**Purification of the M, 92,000 Metalloprotease.** The gelatin degrading M, 92,000 enzyme was purified from conditioned medium of PMA-treated HT1080 cells by sequential gelatin affinity chromatography followed by gel filtration chromatography (Figs. 2 and 3). Although the gelatin-Sepharose column removed the bulk of the conditioned medium proteins after only one passage, no separation of the M, 92,000 and the M, 72,000 gelatinase was achieved.
72,000 species could be achieved with one column (Fig. 2, a and b, Lanes 2). When the pooled material from the first column was applied to a second gelatin-Sepharose column and the bound activities were stepwise eluted with 2, 4, and 7.5% Me2SO, a distinct separation of the M, 92,000 and M, 72,000 enzymes was obtained. The M, 92,000 as well as a M, 120,000 species were greatly enriched in the 2% fractions, while the M, 72,000 species was preferentially found in the 4% and 7.5% fractions (Fig. 2, a and b, Lanes 3–5). A separation between two additional proteins (M, 29,000 and M, 21,000), which coeluted with the gelatinases, also occurred. Both protein species do not possess gelatinolytic activity (Fig. 2b, Lanes 2–5). The M, 29,000 protein, appearing as a broad band on silver stained polyacrylamide gels, largely coeluted with the M, 92,000 gelatinase in 2% Me2SO (Fig. 2a, Lane 3) and was subsequently identified as TIMP by amino-terminal amino acid sequence analysis (see Fig. 4b). In contrast, the M, 21,000 protein mainly coeluted with the M, 72,000 type IV collagenase in 4 and 7.5% Me2SO (Fig. 2a, Lanes 4 and 5). This protein may represent TIMP-2, a recently described metalloprotease inhibitor associated with the M, 72,000 gelatinase/type IV collagenase (23, 24).

Subsequent gel permeation chromatography of the 2% Me2SO eluted pool yielded a symmetrical elution profile of purified M, 92,000 enzyme, when the M, 80,000–120,000 region of the gel filtration column was analyzed by silver stained SDS-PAGE (Fig. 3a, Lanes 1–5). Under nonreducing conditions, a less intense band at approximately M, 120,000 and a diffuse minor band at M, 29,000 representing TIMP (see Fig. 4b) are seen as well. The M, 120,000 band, which forms in variable amounts from preparation to preparation, may represent a complex between TIMP and the latent M, 92,000 enzyme, of which some, but not all, appears to be stable during SDS-electrophoresis (see below). The presence in SDS gels of a M, 29,000 TIMP band, a M, 92,000 band and the M, 120,000 band in fractions corresponding to a M, 80,000–120,000 region of the gel filtration column is consistent with the existence of a TIMP-M, 92,000 complex that is partially dissociated in SDS. The M, 92,000 band observed in the silver stained gel corresponds to a M, 92,000 zone of gelatin lysis observed in the zymographic analysis of the same gel filtration fractions (Fig. 3b, Lanes 1–5) and corresponds to the M, 92,000 enzyme present in conditioned medium (Fig. 3, Lane 6). The gelatinolytic activity of the M, 92,000 enzyme is completely inhibited by EDTA (Fig. 3, Lane 7). The pH optimum of the M, 92,000 enzyme was determined using gelatin zymograms incubated and buffered at pH values from pH 3 to 11. The optimum activity occurred at pH 7.5 (data not shown).

To evaluate possible differences in primary structure between the M, 92,000 and the M, 72,000 progelatinase species, the NH2-terminal amino acid sequence of the M, 92,000 enzyme was determined. Fig. 4a shows the NH2 terminus of the isolated latent M, 92,000 enzyme, which does not share any significant homology with the amino termini of other members of the human metalloprotease family. The sequence is in agreement with the NH2-terminal sequence deduced from a M, 92,000 complementary DNA clone (30).

The M, 92,000 Proenzyme Undergoes Autoactivation. A time course analysis of APMA activation of purified enzyme (Fig. 5a, Lanes 1–5) showed a rapid cleavage of the M, 92,000 proenzyme to a M, 83,000 species with over 50% conversion after 30 min and >90% conversion after 3 h. After 60 min, a second activation product of M, 75,000 appeared, which progressed to become the dominant form after 6 h. The M, 92,000 enzyme, of which some, but not all, appears to be stable during SDS-electrophoresis (see below). The presence in SDS gels of a M, 29,000 TIMP band, a M, 92,000 band and the M, 120,000 band in fractions corresponding to a M, 80,000–120,000 region of the gel filtration column is consistent with the existence of a TIMP-M, 92,000 complex that is partially dissociated in SDS. The M, 92,000 band observed in the silver stained gel corresponds to a M, 92,000 zone of gelatin lysis observed in the zymographic analysis of the same gel filtration fractions (Fig. 3b, Lanes 1–5) and corresponds to the M, 92,000 enzyme present in conditioned medium (Fig. 3, Lane 6). The gelatinolytic activity of the M, 92,000 enzyme is completely inhibited by EDTA (Fig. 3, Lane 7). The pH optimum of the M, 92,000 enzyme was determined using gelatin zymograms incubated and buffered at pH values from pH 3 to 11. The optimum activity occurred at pH 7.5 (data not shown).

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M, 92,000 GELATINASE FROM HT1080 HUMAN TUMOR CELLS

h 92 kDa
APXQRQSTLVLFPGDL

h 72 kDa
APSPIIKFPGDVAPKT

h collagenase
FPATLETQEQDVDLVQ

h stromelysin
YPLDCAARGEDTSMNL

b.

h 29 kDa
XTXVPPHPQTAFCN

h TIMP
CTCVPHPQTAFCN

Fig. 4. Amino terminal amino acid sequence of the M, 92,000 proenzyme and the copurifying M, 29,000 band. Pooled 2% MeSO fractions from the second gelatin-Sepharose column containing 10 µg of M, 92,000 protein and 3 µg of M, 29,000 protein were electrophoresed and electroblotted onto a PVDF membrane. The bands were cut out and sequenced (see "Materials and Methods"). (a) The isolated M, 92,000 proenzyme. For comparison, the amino termini of human (h) M, 72,000 gelatinase/type IV collagenase (5), human interstitial collagenase (2, 3), and human stromelysin (7, 8) are shown. (b) The isolated M, 29,000 band. For comparison, the amino terminal sequence derived from TIMP complementary DNA is shown (20). X, amino acid residues which could not be definitively identified.

proenzyme was completely converted after this time. When purified enzyme was incubated in the presence of 2 mM APMA for 3 h at 37°C and analyzed on silver stained gels, a new major protein product appeared indicating the conversion of the latent M, 92,000 form to the activated M, 83,000 form (Fig. 5a, Lanes 6 and 7). In addition, the M, 120,000 complex disappeared upon APMA treatment and apparently contributed to the M, 92,000 as well as to the M, 83,000 band. A minor M, 75,000 band was also seen. These newly formed protein products correspond to the APMA generated zones of lysis in the zymogram (Fig. 5a, Lane 4).

The incubation of purified M, 92,000 zymogen with 2 mM APMA for 3 h in the presence of 5 mM EDTA completely prevented any activation (Fig. 5b, compare Lanes 2 and 3), while the presence of 2 mM PMSF, a serine protease inhibitor, had little effect except for a reduction in the appearance of the M, 75,000 species (Fig. 5b, Lane 4). The inhibition of conversion by EDTA with a purified preparation of a metalloenzyme suggests the action of the enzyme upon itself. This is compatible with a physiological mechanism of autoactivation via intermolecular and/or intramolecular action and would be analogous to the proposed activation mechanism of the M, 72,000 type IV collagenase (19, 38).

Inhibitor studies with purified, activated enzyme in which the degradation of soluble ³H-gelatin was monitored indicated that the M, 92,000 enzyme is a zinc metalloprotease since 1,10-phenanthroline inhibited it totally (100%) (Table 1). Although 5 mM EDTA yielded an inhibition of only 80% in this experiment, the same concentration of EDTA completely inhibited gelatinolysis on zymograms (Fig. 3c, Lane 7). This slight differential effect of EDTA might be due to the two different assays used.

The M, 92,000 Gelatinase from HT1080 Cells Is Closely Related to the Neutrophil Derived Gelatinase. A partially purified M, 92,000 gelatinase preparation, which did not contain the M, 120,000 complex but did contain some M, 72,000 enzyme, as evidenced in a silver stained SDS gel (Fig. 6a, Lane 1) was recognized by an anti-neutrophil gelatinase antibody in a Western immunoblot yielding a distinct reaction at M, 92,000 (Fig. 6b, Lane 2), thereby demonstrating their close immunological relationship. The antibody did not recognize the M, 72,000 enzyme in the preparation. Purified M, 72,000 type IV collagenase also was not recognized by the antibody (data not shown).

A mouse antibody raised against partially purified M, 92,000 enzyme from HT1080 cells also recognized only the M, 92,000...
enzyme when immunoblotted against the same preparation (Fig. 6b, Lane 1) yielding an immunoreaction identical to the anti-neutrophil gelatinase antibody (Fig. 6b, Lane 2). This result demonstrates the immunological relationship between the neutrophil M, 97,000 gelatinase (31) and the HT1080 M, 92,000 gelatinase. When the anti-neutrophil gelatinase antibody was reacted with a preparation of M, 92,000 enzyme which contained a small amount of M, 120,000 complex (Fig. 6a, Lane 2), the M, 120,000 species yielded a faint but distinct immunoreactivity (Fig. 6b, Lane 3). When this antibody was reacted with a FPLC purified preparation of M, 92,000 enzyme which contained a greater amount of M, 120,000 complex as in Fig. 5a, Lane 6, a significant immunoreaction was evident at M, 120,000 (Fig. 6b, Lane 4). These results indicate that the M, 120,000 species represents a SDS stable complex composed of M, 92,000 enzyme determinants and possibly TIMP. Further evidence that the M, 120,000 species might be a M, 92,000/TIMP complex is indicated in Fig. 6b, Lane 5, in which the FPLC purified preparation was immunoblotted with an anti-TIMP antibody. An intense immunoreaction occurs at the M, 29,000 position corresponding to the position of free TIMP while a faint but distinct immunoreaction occurred at the M, 120,000 position. Reduction of the FPLC purified preparation with β-mercaptoethanol (Fig. 6a, Lane 3) eliminates the M, 120,000 species and yields a single protein band at M, 92,000 and a diffuse, faint protein band at 29 kDa. We have observed that under reducing conditions TIMP is less intensely stained with silver. The M, 92,000 and 29,000 bands migrate slightly slower under the reducing conditions, as do many disulfide containing proteins due to unfolding of the molecules.

Substrate Specificities of the M, 92,000 Metalloprotease. As already shown by the lysis of gelatin substrate gels, the M, 92,000 activated enzyme is a highly effective gelatinase (Figs. 1, 3, and 5). Incubation of purified M, 92,000 gelatinase (activated by 2 mM APMA) with type I collagen derived gelatin at 23°C brings about a rapid digestion of gelatin into small peptides (Fig. 7, Lanes 2 and 3) and completely degrades the gelatin at 37°C (Fig. 7, Lane 4). Type V collagen derived gelatin is equally well degraded (data not shown). The latent, nonactivated M, 92,000 form is completely inactive towards gelatin (Fig. 7, Lane 5).

Soluble native type V collagen is slowly, but effectively, cleaved at a single site by the M, 92,000 enzyme which had been cut out from gels and activated by APMA. The cleavage by activated enzyme creates two large α1(V) and α2(V) fragments (Fig. 8a). Soluble, native type IV collagen is less effectively digested by the activated M, 92,000 enzyme and even after 48 h, only a minor portion of the initial amount of type IV collagen disappeared without producing any distinct cleavage fragments (Fig. 8b). Assays using purified activated M, 92,000 enzyme taken directly from FPLC fraction 22 (Fig. 3) also indicated that type V collagen was hydrolyzed while type IV collagen was only marginally degraded (data not show). The M, 92,000 enzyme is completely ineffective against soluble, native type I collagen (Fig. 8c). Among the glycoproteins found in basement membrane, fibronectin and laminin are resistant to digestion.

Table 1: Inhibition of [3H]-labeled gelatin digestion by purified M, 92,000 enzyme

After the M, 92,000 enzyme (10 μl) was preactivated with 2.5 mM APMA for 2 h at 37°C, the inhibitors were included in the reaction mixture at a final concentration as indicated and assayed for the degradation of [3H]-gelatin for 4 h at 37°C as described in “Materials and Methods.”

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<th>Inhibitor</th>
<th>Concentration (mM)</th>
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Fig. 6. Western immunoblot analysis of the M, 92,000 gelatinase and the M, 120,000 complex. A preparation containing both M, 92,000 and M, 120,000 enzyme but no M, 120,000 complex (a, Lane 1, b, Lanes 1 and 2) as well as preparations containing M, 92,000 enzyme and M, 120,000 complex (a, Lanes 2 and 3; b, Lanes 3, 4, and 5) were electrophoresed on 10% SDS-PAGE and silver stained (a) and immunoblotted (b). a, Lanes 2 and 3, 50 μl of an identical gelatinase preparation without reduction (Lane 2) and with reduction (Lane 3). The partially purified preparation shown in a, Lane 1 was reacted with anti-M, 92,000 tumor gelatinase (b, Lane 1) and with anti-M, 97,000 neutrophil gelatinase (b, Lane 2) demonstrating their immunological relationship. Both antisera do not cross-react with M, 72,000 gelatinase which is present in the preparation. The material shown in a, Lane 2 was reacted with anti-M, 97,000 neutrophil gelatinase (b, Lane 3) yielding a strong M, 92,000 band as well as a faint but distinct band at M, 120,000 which intensifies when a FPLC purified preparation with greater amounts of M, 120,000 is used (b, Lane 4). An antibody against human TIMP recognizes the M, 29,000 as well as the M, 120,000 band (b, Lane 5). βME, β-mercaptoethanol.
of the matrix metalloprotease family. The enzyme is secreted as an inactive zymogen, works optimally at physiological pH, forms a complex with TIMP in the latent stage, and autoactivates upon organomercurial treatment to a $M_r$, 83,000 and subsequent $M_r$, 75,000 form. The enzyme shows little or no hydrolysis of noncollagenous matrix proteins. It rapidly degrades gelatinized (denatured) collagens at 23°C and 37°C. The enzyme also cleaves slowly, but efficiently, native type V collagen at 32°C. The $M_r$, 92,000 gelatinase, however, exhibited only poor type IV collagen degrading activity. Since the presence of small amounts of TIMP in our isolated preparation might have altered the relative catalytic activity of the enzyme toward type V and IV collagens, we have utilized the approach described by Hibbs et al. (6), in which a single band of $M_r$, 92,000 enzyme, free of TIMP, is sliced from SDS gels and incubated with purified type V and IV collagens. Under these conditions, type V collagen is cleaved (Fig. 8a), while type IV collagen is only minimally degraded even after 48 h of incubation (Fig. 8b). Gelatin, however, is cleaved in as short a time as 2 h with 100-fold less enzyme (Fig. 7). For these reasons we prefer at this point not to call the $M_r$, 92,000 enzyme a “type IV collagenase” but believe that the enzyme functions mainly as a gelatinase. Wilhelm et al. (30) reported that the $M_r$, 92,000 enzyme isolated from SV40 transformed fibroblast was a type IV collagenase. The reason for these discrepant results are unclear.

The enzyme preparation which we have isolated contains the inhibitor of metalloproteases, TIMP, as indicated by its apparent molecular weight and its homologous amino terminal sequence (Fig. 4b). TIMP has been shown to inhibit the activated form of interstitial collagenase, which represents the prototype of the metalloproteinases, by forming a 1:1 complex but does not bind to the latent interstitial collagenase (39). Our results with gel filtration chromatography indicate that TIMP forms a noncovalent complex with the latent $M_r$, 92,000 gelatinase, which may represent the actual form of the enzyme as it is present in conditioned medium. This complex between secreted proenzyme and TIMP, usually cosecreted by the same cells, has recently been observed by others (30) and may represent a natural mode of negative regulation at the zymogen level. Furthermore, TIMP-2 molecule ($M_r$, 21,000) has been described which forms a 1:1 complex with latent $M_r$, 72,000 type IV collagenase/gelatinase (23). In this respect, gelatinases may represent a distinct subgroup within the metalloprotease family. How and when these complexes dissociate under physiological conditions yielding active enzyme is unknown at present, since the natural activator(s) of the $M_r$, 72,000 and the $M_r$, 92,000 gelatinases has yet to be identified. Plasmin, generated through the activity of plasminogen activator, has been shown to activate interstitial collagenase and stromelysin (15) but is inefficient at activating the $M_r$, 72,000 and $M_r$, 92,000 gelatinases. Our finding of an additional $M_r$, 120,000 zymogen/TIMP complex which is SDS stable and immunoreactive with both anti-$M_r$, 92,000 antibody and anti-TIMP antibody but dissociates upon reduction and APMA treatment is somewhat surprising. We speculate that it might represent the exchange of disulfide bonds from intramolecular positions within enzyme and TIMP to an intermolecular position between the two molecules. The complex might be created during the concentration step of conditioned medium and is carried through the purification procedure by virtue of the binding of the complex to gelatin-Sepharose. The dissociation of the complex upon treatment with β-

**DISCUSSION**

We have isolated and characterized a $M_r$, 92,000 gelatinase from human tumor cells, which represents a distinct member of the matrix metalloprotease family.
mercaptoethanol (Fig. 6a, Lane 3) indicates that some disulfide bond(s) is involved in the structure of the SDS stable complex. Interestingly, APMA activated neutrophil gelatinase also forms a complex with TIMP which is SDS stable under nonreducing conditions (40).

The actual physiological role of the secreted M, 92,000 enzyme and its involvement in normal matrix turnover is an unresolved question at this point. The enzyme, once activated and free of TIMP, may operate within the connective tissue compartment as well as in the basement membrane compartment of the extracellular matrix. In connective tissue, the enzyme might synergistically degrade denatured collagen fibrils which are generated initially by the action of interstitial collagenase (40). This would be consistent with the dominant catalytic activity of the M, 92,000 enzyme in vitro, namely that of a gelatinase. The M, 92,000 enzyme also cleaves native type V collagen, which is a triple helical molecule usually composed of $\alpha_1(IV), \alpha_2(IV)$ chains. Small amounts of type V collagen have a widespread tissue distribution similar to type I collagen but it also occurs as a structural component of basement membrane (41). There is evidence that type V collagen is copolymerized within type I collagen and forms hybrid fibrils, suggesting a close cooperation between the two collagen types (42). However, a specific function for type V collagen in the connective tissue space has yet to be determined, and thus the role of the M, 92,000 enzyme as an interstitial type V collagenase is not yet established.

The M, 92,000 gelatinase most likely can degrade basement membrane type V collagen, since the latter has the same structural composition as the interstitial form (43). A limited role of the M, 92,000 gelatinase in basement membrane remodeling may be significant in some physiological and pathological circumstances. Neutrophils and monocytes/macrophages, cells with a high invasive and migratory capacity, constitutively secrete a M, 92,000 gelatinase which is similar if not identical to the HT1080 tumor cell gelatinase which we have isolated, based on immunological evidence and substrate profile (6, 40, 44). Thus the M, 92,000 gelatinase may be utilized by migrating neutrophils and macrophages as they penetrate basement membrane and extracellular matrix during an inflammatory response. For tumor cells, the acquisition of newly expressed or overexpressed M, 92,000 gelatinase seems to be correlated with the invasive and metastatic phenotype. ras oncogene transfection into mouse NIH/3T3 parental cells, which produce only M, 72,000 gelatinase, induced the M, 92,000 gelatinase and conferred a metastatic phenotype on these cells (45). This finding is in agreement with our results which show a significantly higher baseline production of M, 92,000 in the highly invasive HT1080 fibrosarcoma cells as compared to skin fibroblasts and the increased production of M, 92,000 enzyme observed in the highly metastatic variant of HEP-3 cells (Fig. 1).

The increase in M, 92,000 gelatinase expression by PMA treatment of tumor cells and normal fibroblasts (Fig. 1) indicates that the gene for this enzyme may be a member of the phorbol ester inducible genes, a process that involves the activation of protein kinase C (46). Other targets of PMA induction include the collagenase and stromelysin genes which also have been linked to tumor invasiveness (2, 16, 47). Although normal fibroblasts secrete enhanced levels of metalloproteinases upon PMA treatment, interestingly, the secretion of TIMP by human lung fibroblasts is also stimulated by PMA (32). These results suggest that not only the amount of proteinases produced but also the balance between enzyme levels and inhibitor levels may dictate the relative invasiveness of a given cell in vivo. Consistent with this notion is the finding of an inverse correlation between TIMP levels and the invasive potential of a series of tumor cell lines (48). Also, a 50% reduction of TIMP expression by transfection with antisense TIMP RNA confers oncogenicity to Swiss 3T3 cells (49).

The unexpectedly high secretion of M, 92,000 enzyme in human synovial fibroblasts compared to the low level observed in skin fibroblasts (Fig. 1) suggests that normal fibroblasts are subjected to differential regulation of their metalloproteinase expression depending on their tissue of origin. Murphy et al. (31) found significant M, 92,000 gelatinase expression in rabbit synovial fibroblasts and rabbit chondrocytes but no M, 92,000 production in human gingival fibroblasts.

Similarly, normal epithelial cells do not produce any gelatinase while H-ras oncogene transformation induces the M, 72,000 gelatinase in bronchial epithelial cells and confers the ability to degrade type IV and V collagen in vitro (5). Our results show an enhanced M, 92,000 secretion in high metastatic versus low metastatic HEP-3 epithelial cells. Yamagata et al. (29) found a significant increase of M, 92,000 gelatinase in high metastatic versus low metastatic variants of murine colon carcinoma cells as well as Lewis lung carcinoma cells. Future experiments will have to address the molecular mechanism behind these correlations and to define the regulatory defect in the expression of M, 92,000 enzyme in malignant tumor cells. Furthermore, the relationship of the M, 92,000 to the M, 72,000 gelatinase and their relative importance in the invasive and metastatic phenotype will have to be examined using both transfection techniques and specific antibodies against the different enzymes.

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